National Library of Canada

Acquisitions and Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada

Direction des acquisitions et des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

UNIVERSITY OF ALBERTA

# Amino Acid Alkylation; Synthesis of Diaminopimelic Acid (DAP) and Derivatives; Inhibition Studies on *N*-Succinyl-DAP Aminotransferase

BY

William Aubrey Sherwin

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

DEPARTMENT OF CHEMISTRY

Edmonton, Alberta

Spring 1994

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

**The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.**

**L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.**

**The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.**

**L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.**

ISBN 0-612-11371-X

Canada

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: William Aubrey Sherwin

TITLE OF THESIS: Amino Acid Alkylation; Synthesis of Diaminopimelic Acid (DAP) and Derivatives; Inhibition Studies on *N*-Succinyl-DAP Aminotransferase

DEGREE: Doctor of Philosophy

YEAR THIS DEGREE GRANTED: Spring 1994

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

(SIGNED)..W.A.Sherwin.....

PERMANENT ADDRESS:

37 Guilfoyle St

Yarralumla, A.C.T. 2600

Australia

DATED: October 26, 1993

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Amino Acid Alkylation; Synthesis of Diaminopimelic Acid (DAP) and Derivatives; Inhibition Studies on *N*-Succinyl-DAP Aminotransferase submitted by William Aubrey Sherwin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr John C. Vederas (Supervisor)

Dr. Karl R. Kopecky

Dr George Kotovych

Dr. William A. Ayer

Dr. Susan Jensen

Dr. Ronald J. Parry (External)

Date: 25 October, 1993

To Gwen and my mother

## Abstract

The α-alkylation of *N*-benzoyl α-amino acid esters of 3α,12α-dihydroxy-23-nor-5β-cholane (**12**) (obtained by decarboxylation of deoxycholic acid (**2**)) was investigated as a new route to the stereoselective synthesis of α-amino acids. Alkylation of the sterol 3α-esters of glycine (**13**), alanine (**14**, **15**), and phenylalanine (**16**) with methyl iodide or benzyl bromide showed little or no stereoselectivity.

Stereoselective synthesis of the diaminopimelic acid (DAP) stereoisomers was investigated. Evans' use of trisyl azide (**25**) for stereoselective azide transfer to chiral enolates was applied to the chiral bis-phenylcyclohexyl ester of pimelic acid (**24**). However, enolization of **24** resulted in its cyclization rather than azide transfer from **25**. An improved route to the DAP stereoisomers by stepwise dialkylation of diiodopropane, initially with Seebach's *N*-benzoylimidazolidinone, and subsequently with Schöllkopf's bis-lactim ether, gave the diprotected D,D-DAP derivative (**37**) in 35% yield. Selective hydrolysis of the bis-lactim ether gave the mono-protected DAP derivative (**38**) in 67% yield. Hydrolysis of **37** in 6 N HCl gave D,D-DAP (**39**) in partially epimerized form. A short route to L,L- or D,D-DAP was developed by one-step dialkylation of 1,3-dibromopropane with Seebach's *N*-Boc-imidazolidinone (**43**). The bis-imidazolidinone (**44**) was hydrolyzed with 6 N HCl at 100 °C to D,D-DAP (**39**) in 95% d.e.

The pyridoxal phosphate-dependent *N*-succinyl-DAP aminotransferase enzyme was isolated from wild type *E. coli* with 89-fold purification, with a maximum specific activity of 61 Units/mg. The most efficient purification steps were dye-ligand and hydroxyapatite chromatography. Values for $K_m$ of 0.2 mM for (6*S*)-6-*N*-succinylamino-2-oxopimelate (**50**), the α-keto substrate, and 1.2 mM for glutamate, as well as a value for $V_{max}$ of 65 nmol/min·mg in Tris pH 8, at 30 °C, were determined by a spectrophotometric assay coupled to glutamate dehydrogenase, measuring the conversion of **50** to *N*-succinyl-DAP. The α-keto substrate **50** was prepared as the tri-lithium salt

via a $FeCl_3$-catalyzed ene reaction of methyl glyoxylate (**46**) with *N*-succinylallylglycine dimethyl ester (**45**). The racemic des-6-amino (**5**) and des-7-carboxy (**6**) α-keto substrate analogs were prepared via a similar ene reaction with **46**. The 2-*N*-amino analog **3** of 6-*N*-succinyl-DAP, the enzyme's α-amino product, was prepared from **50** by hydrazone formation with hydrazine and reduction to the hydrazino derivative. Compounds **5** and **6** were not inhibitors of the aminotransferase, however **5** was a weak substrate. Compound **3** was a strong inhibitor of the enzyme (>99%) at a concentration of 1 mM.

**Acknowledgements**

I would like to thank my supervisor, Dr. John C. Vederas, for his excellent guidance and support during my studies. The following people are gratefully acknowledged for their collaborative work: Dr. Lister K. P. Lam, Dr. Russell J. Cox and Professor Michael A. Pickard. I would like to thank all the members of our research group, past and present, for helpful discussions, in particular: Dr. Gregory Helms, Dr. Patricia Lane-Bell, Dr. T. Mark Zabriskie, Mr. Aubrey J. Mendonca and Dr. Michael Klinge. My thanks also go to Professor Monica M. Palcic and her research group for helpful advice. Special thanks to Dr. Christopher Lowe for proof-reading this manuscript.

The administrative, spectral, and technical staff in the Department of Chemistry are gratefully acknowledged for their excellent and friendly assistance. Finally, I would like to thank my mother, Audrey, and my wife, Gwen, for their unfailing support and encouragement.

## Table of Contents


| | | Page |
|---|---|---|
| 1. | **Introduction** ..... | 1 |
| 2. | **Results and Discussion** ..... | 17 |
| | Stereoselective Alkylation of Chiral α-Amino Acid Esters ..... | 17 |
| | Synthesis of the Diaminopimelate Isomers and Their Derivatives: | |
| | 1. Stereoselective azidation of chiral ester enolates ..... | 23 |
| | 2. Stereoselective alkylation of chiral glycine synthons ... | 28 |
| | Studies on *N*-Succinyl Diaminopimelate Aminotransferase: | |
| | Preparation of the enzyme's α-keto substrate ..... | 39 |
| | 2-*N*-Amino- and 2-*N*-hydroxy-6-*N*-L-succinyl- -diaminopimelate ..... | 41 |
| | Desamino and descarboxy α-keto substrate analogs ..... | 43 |
| | Activity and protein assays ..... | 46 |
| | Isolation of the aminotransferase ..... | 48 |
| | Determination of Michaelis-Menten constants ..... | 55 |
| | α-Keto substrate analog studies ..... | 59 |
| 3. | **Experimental** ..... | 62 |
| 4. | **References** ..... | 114 |

## List of Tables


| Table | | Page |
|---|---|---|
| 1. | Antibiotics which target peptidoglycan biosynthesis..... | 5 |
| 2. | *N*-Hydroxy- and *N*-amino-α-amino acid analogs as inhibitors of PLP-dependent enzymes ..... | 15 |
| 3. | Esterification results ..... | 20 |
| 4. | Alkylation results ..... | 21 |
| 5. | Results of the dialkylation route to DAP ..... | 29 |
| 6. | Aminotransferase purification summary ..... | 54 |
| 7. | Michaelis-Menten constants ..... | 57 |
| 8. | Effects of α-keto substrate analogs on enzyme activity ..... | 59 |
| 9. | Inhibition results ..... | 61 |

## List of Figures


| Figure | | Page |
|---|---|---|
| 1. | Alkylation of chiral Schiff bases ..... | 2 |
| 2. | Proposed stereoselective alkylation of chiral enolates ..... | 4 |
| 3. | Peptidoglycan monomer (*E. coli*) ..... | 6 |
| 4. | Peptidoglycan structure in *E.coli* ..... | 7 |
| 5. | Bond cleavages catalyzed by PLP-dependent enzymes ..... | 13 |
| 6. | Stable PLP complexes formed with amino acid analogs ..... | 16 |
| 7. | Sterol esters of *N*-benzoyl-protected amino acids ..... | 17 |
| 8. | Esterification reagents ..... | 19 |
| 9. | Proposed stereoselective azidation route to the DAP isomers ..... | 24 |
| 10. | Epimerization of **31** via keto-enol tautomerization ..... | 27 |
| 11. | Chiral glycine synthons of Seebach and Schöllkopf ..... | 28 |
| 12. | Mechanism of $^{2}H$ incorporation into **50** ..... | 41 |
| 13. | Coupled assay for *N*-succinyl-DAP aminotransferase ..... | 46 |
| 14. | Aminotransferase isolation scheme ..... | 49 |
| 15. | Cibacron Blue 3G-A-Agarose Type 3000-CL ..... | 50 |
| 16. | Dye-ligand binding study ..... | 52 |

17. Silver stained SDS-PAGE gels of aminotransferase isolation ..... 53

18. Lineweaver-Burk plots ..... 56

19. $K_m$ and $V_{max}$ replots ..... 58

20. 2-Hydroxytetrahydropyran-2,6-dicarboxylate (2-HTHP) ..... 60

## List of Abbreviations

| | |
|---|---|
| $\Delta A_{340}$ | change in optical absorbance at 340 nm |
| Ac | acetyl |
| Ala | alanine |
| Anal. | analysis |
| Ar | aryl |
| b | broad |
| Bn | benzyl |
| Boc | *tert*-butoxycarbonyl |
| BOP-Cl | *N,N*-bis-(2-oxo-3-oxazolidinyl)-phosphinic chloride |
| bp | boiling point |
| Bu | butyl |
| Calcd | calculated |
| CBz | benzyloxycarbonyl |
| CDI | 1,1'-carbonyldiimidazole |
| CI | chemical ionization |
| d | doublet |
| DAP | diaminopimelic acid |
| DCC | 1,3-dicyclohexylcarbodiimide |
| d.e. | diastereomeric excess |
| DEAE | diethylaminoethyl |
| DMPU | *N,N'*-dimethylpropyleneurea |
| DMSO | dimethylsulfoxide |
| $\varepsilon$ | extinction coefficient |
| EDTA | ethylenediaminetetraacetic acid |
| e.e. | enantiomeric excess |

| | |
|---|---|
| Et | ethyl |
| FAB | fast atom bombardment |
| GlcNAc | *N*-acetylglucosamine |
| Glu | glutamate |
| h | hour |
| HMDS | hexamethyldisilazane |
| HPLC | high pressure liquid chromatography |
| 2-HTHP | 2-hydroxytetrahydropyran-2,6-dicarboxylate |
| IR | infra red |
| kD | kilodalton |
| $K_{eq}$ | equilibrium constant |
| $K_m$ | Michaelis-Menten constant |
| LDA | lithium diisopropylamide |
| lit. | literature |
| m | multiplet |
| Me | methyl |
| MES | 4-morpholineethanesulfonic acid |
| min | minute |
| MOM | methoxymethyl |
| MOPS | 3-(*N*-morpholino)propanesulfonic acid |
| mp | melting point |
| MPLC | medium pressure liquid chromatography |
| MS | mass spectrometry |
| MurNAc | *N*-acetylmuramic acid |
| M.W. | molecular weight |
| $NADP^+$ | nicotinamide adenine dinucleotide phosphate, oxidized form |
| NADPH | nicotinamide adenine dinucleotide phosphate, reduced form |

| | |
|---|---|
| NMR | nuclear magnetic resonance |
| OP | $-OPO_3H$ |
| Ph | phenyl |
| PLAP | pig liver acetone powder |
| PLP | pyridoxal 5-phosphate |
| PMSF | phenylmethylsulfonyl fluoride |
| posFAB | positive ion fast atom bombardment |
| $^{i}Pr$ | isopropyl |
| q | quartet |
| $R_f$ | retention factor |
| RP | reverse phase |
| s | singlet |
| S | substrate |
| SDS-PAGE | sodium dodecylsulfate-polyacrylamide gel electrophoresis |
| THDP | tetrahydrodipicolinate |
| THF | tetrahydrofuran |
| TLC | thin layer chromatography |
| *p*-tosyl | *p*-toluenesulfonyl |
| t | triplet |
| Tris-HCl | tris-(hydroxymethyl)aminomethane-hydrochloride |
| UV | ultraviolet |
| V | velocity |
| $V_{max}$ | maximal enzyme velocity |

## INTRODUCTION

Non-proteinogenic α-amino acids are important in numerous scientific disciplines as well as the associated pharmaceutical, agricultural and food industries. Some of their applications include: incorporation into enzyme inhibitors; tools for studying enzyme mechanisms; functional and structural modifiers of biologically-active peptides; and chiral starting materials for chemical synthesis. Consequently demand for these amino acids, especially unnatural and rare natural compounds, has sparked a need for new general stereoselective syntheses to supply them. The last decade has seen the development of various elegant methods for the stereospecific preparation of a wide variety of α-amino acid derivatives. These methods have recently been surveyed in a recent symposium-in-print[1] and in an excellent review.[2] A number of other reviews covering earlier and achiral methods have previously appeared.[3-5]

The more recent stereoselective methodology can be grouped into the following approaches: reaction of nucleophilic chiral glycine equivalents with alkyl halides, aldehydes, and other electrophiles; reaction of glycine carbocation equivalents with various nucleophiles; electrophilic α-amination of chiral enolates or nucleophilic amination of α-substituted acids; heterogeneous hydrogenation of chiral dehydroamino acids and asymmetric homogeneous hydrogenation of achiral dehydroamino acids; homologation at α–, β- or γ-carbons; asymmetric Strecker syntheses; and enzymatic syntheses, both via resolution of racemates and catalysis of stereoselective reactions of prochiral substrates. A detailed review of these areas by Williams[2] is available. Relevant areas will be discussed separately in the following sections.

Alkylation at the α-position of chiral glycine derivatives usually employs heterocycles (piperazines, imidazolidinones, oxazolidinones and oxazolines) or chiral Schiff bases of glycine.[2] Examples wherein the glycine is in the open chain form usually involve metal complexation to provide a conformation of the amino acid with restricted access for approaching electrophiles. For instance Belokon and colleagues[6] employed chiral Schiff

bases of glycine or alanine in a square planar complex with $Ni^{2+}$ (Figure 1) with shielding of one face of the enolate by an *N*-benzyl group to obtain alkylated amino acids with up to 92% e.e. Chelation with non-transition metals has also been used to maintain a chiral conformation for the purpose of stereoselective amino acid synthesis. Evans and co-workers have obtained very high diastereoselectivities in the addition of various

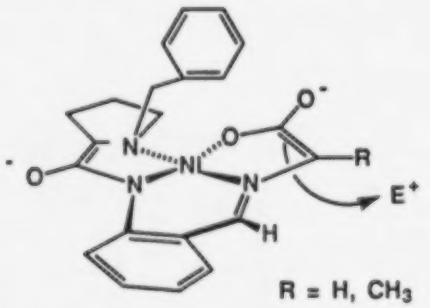
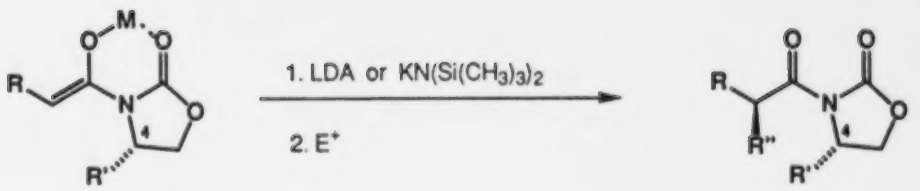



**Figure 1** Alkylation of chiral Schiff bases

electrophiles to chiral carboximides (Scheme 1).[7] It is proposed that metal chelation of the enolate and carboximide carbonyl oxygens prevents formation of the *E* enolate and thereby leads to only one stereoisomer because of steric hindrance by the 4-substituent on the oxazolidinone. This substituent also blocks the approach of electrophiles from the *re* face (when R' = alkyl). The synthesis of amino acids by this route has been achieved with several different N-electrophiles. For example, treatment of the lithium enolate with di-*tert*-butyl azodicarboxylate (DBAD) affords the hydrazide, which after deprotection and hydrogenolysis with Raney nickel gives the free amino acids in up to >99% e.e.[7,8] Evans and Britton[9] also used 2,4,6-triisopropylbenzenesulfonyl azide (trisyl azide) with such enolates (R = Bn, M = K), to obtain azide transfer with diastereoselectivites of up to >99% d.e. The free amino acids were subsequently obtained by hydrolysis of the carboximide and hydrogenolysis of the azide.

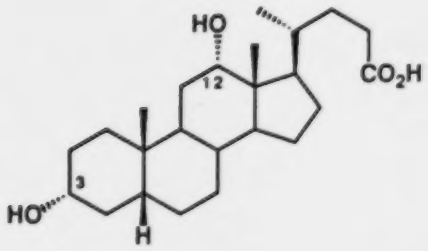



| R' | M | E+ | R" |
|---|---|---|---|
| Bn, $^{i}$Pr | Li | $^{t}$BuO$_2$C-N=N-CO$_2$$^{t}$Bu | N(CO$_2$$^{t}$Bu)NHCO$_2$$^{t}$Bu |
| Bn | K | 2,4,6-triisopropylbenzene-sulfonylazide | $N_3$ |

**Scheme 1**

We envisaged that attachment of an amino acid to a chiral template either covalently or via metal chelation (transiently) could then allow stereoselective alkylation due to selective protection of one face of the enolate from the approaching electrophile. Deoxycholic acid (**1**), a naturally occurring bile acid, was chosen as the source of the chiral template for this study as it is readily available and relatively inexpensive ($200/kg). Due to the cis-fused A/B ring junction an N-protected amino acid esterified to the 3α-hydroxyl could be favorably positioned to be held underneath the B and C rings by metal chelation to the 12α hydroxyl oxygen. Examination of molecular models of *N*-benzoyl protected amino acid esters as the enolate-dianion intermediates suggests the enolate double bond should be approximately co-planar with the sterol skeleton to allow unhindered Li-chelation of the 12-alkoxy group and the benzoyl oxygen as shown in Figure **2**. This allows the phenyl group to be held away from the sterol and also avoids major interactions with the sterol's axial α-hydrogens. An electrophile could then approach from one face of the enolate double bond. The use of a substituted amino acid would allow the introduction of a second α-substituent providing a route to α,α-disubstituted amino acids. These types of compounds have become increasingly important in medicine and biochemistry, since they

provide conformational restriction (eg. 1-aminocyclohexanecarboxylic acid[10]) and protease resistance in physiologically active peptides. They also display potent enzyme inhibition (eg. 2-(hydroxymethyl)aspartic acid is an inhibitor of aspartate aminotransferase[14]).[11-14] The use of deoxycholic acid as the source of the sterol template requires that the side chain carboxylate functiontionality be removed to prevent it from interfering in the complexation of the dianion intermediate.




1

**Deoxycholic acid**

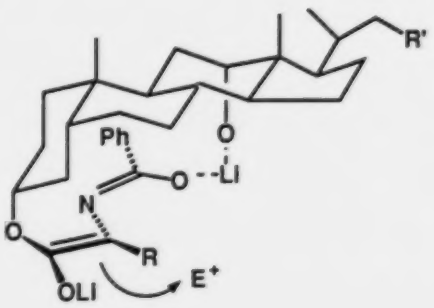


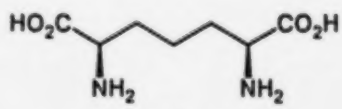

**Figure 2** Proposed stereoselective alkylation of chiral enolates

The development of bacterial resistance to many of the presently used antibiotics including the β-lactams and the glycopeptides,[15] as well as the disturbing re-emergence of resistant strains of previously controlled diseases, such as tuberculosis, has put renewed pressure on the race to develop new antibiotics. Fortunately, the rapid advance of knowledge and techniques in the biological sciences, particularly in the area of molecular biology, has greatly simplified the study of individual metabolic enzymes from pathogenic organisms, allowing the rational design of specific inhibitors. Ideally, a knowledge of the enzyme's active site and mechanism of action will allow the design of transition state analogs that will be more efficient and selective inhibitors. By targeting metabolic pathways or structural features of the pathogen that do not occur in humans, cytotoxicity should be minimal or nonexistent. The peptidoglycan layer of the bacterial cell wall is such

**Table 1** Antibiotics which Target Peptidoglycan Biosynthesis[15,16]

| Target | Antibiotic |
|---|---|
| Alanine racemase | (1-Aminoethyl)-phosphonic acid (Ala-P) |
| | Alafosfalin, D-Cycloserine |
| D-Ala-D-Ala Ligase | D-Cycloserine |
| GlcNAc enolpyruvyl transferase | Phosphonomycin |
| Translocation across cytoplasmic membrane | Bacitracin, Tunicamycin |
| Transpeptidation of peptidoglycan | β-Lactams |
| Binding of peptidyl D-Ala-D-Ala | Vancomycin |

a target and is the site of action of numerous antibiotics (Table **1**). This net-like polymer provides rigidity to the cell wall and protects the cell from lysis due to its high internal osmotic pressure. It consists of a matrix of glycan chains of alternating β-1,4-linked *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) substituted by pentapeptide chains at the D-lactyl groups of the MurNAc residues (Figure **3**). The composition of these peptides and the manner and degree of their crosslinking varies considerably between species, particularly for Gram positive bacteria where the peptidoglycan layer is much more dominant than in Gram negative bacteria, constituting up to 80% of the cell wall. The sequence of the pentapeptide is L-Ala-γ-D-Glu-X-D-Ala-D-Ala where X is often L-lysine in Gram positive bacteria and usually D,L-diaminopimelate (*meso*-DAP, **2**) in Gram negative bacteria (Figure **4**). The crosslinking of the peptide




**2**

*meso*-DAP

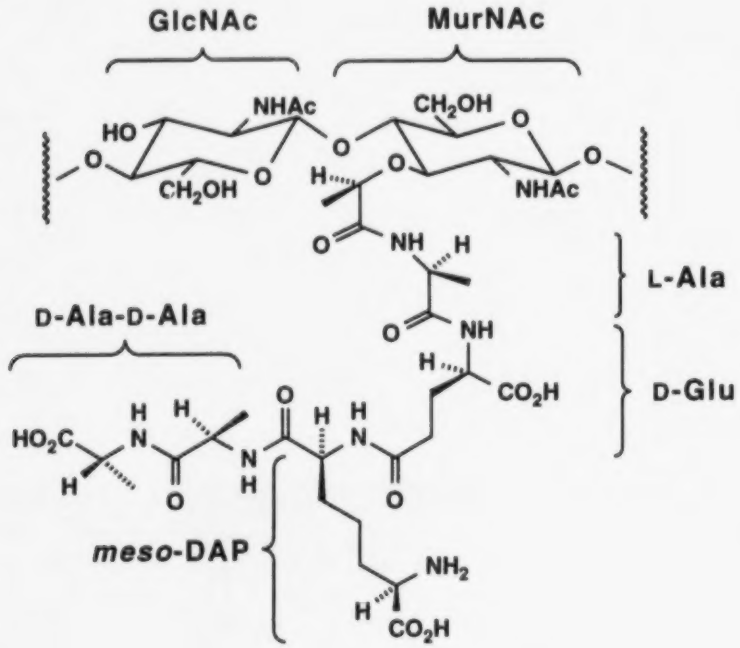



**Figure 3** Peptidoglycan monomer (*E.coli*)

chains often occurs through peptide bond formation of the ω-amino group of X with the penultimate D-Ala, eliminating the terminal D-Ala in the process. Alternatively crosslinking can occur through an additional short peptide (< 6 amino acids) in some Gram positive bacteria.[16-18] In addition there are some examples of Gram negative bacteria where *meso*-DAP is replaced by D,D-DAP,[19-23] L,L-DAP,[17,24] 3-hydroxy-DAP,[25,26] and lanthionine,[27] as well as more complex crosslinks with 2 or more DAP residues.[17,28] Lipoproteins also attach to peptidoglycan by peptide bond formation of a lysine ω-amino with the free carboxyl of *meso*-DAP.[29,30] The pentapeptide sequence is not gene-encoded but depends upon the amino acid specificity of the ligases which synthesize the peptidoglycan precursors.[15]

While DAP is not biosynthesized in mammals, compounds containing DAP, which resemble peptidoglycan fragments (muramyl peptides), have been found in human urine

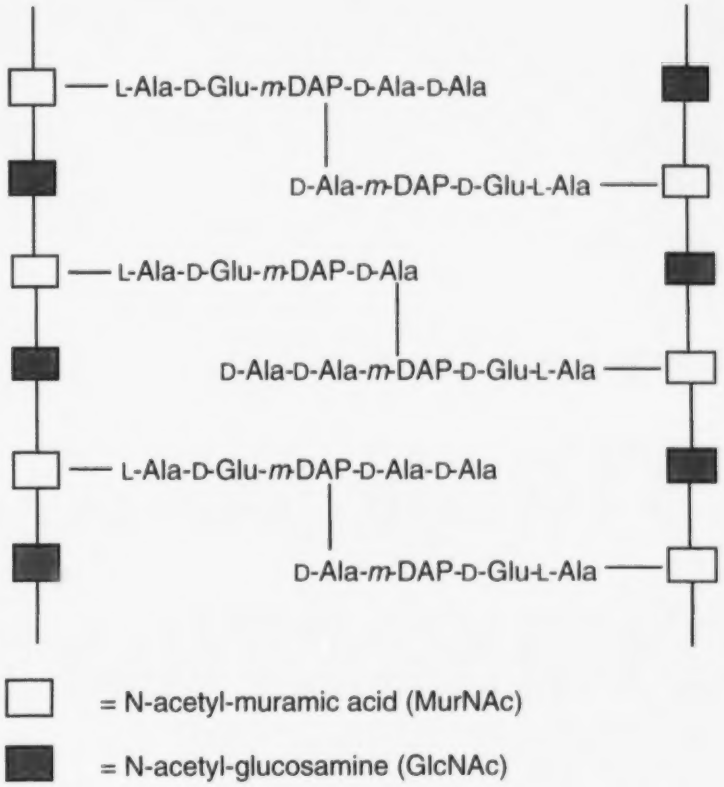



**Figure 4** Peptidoglycan structure in *E.coli*

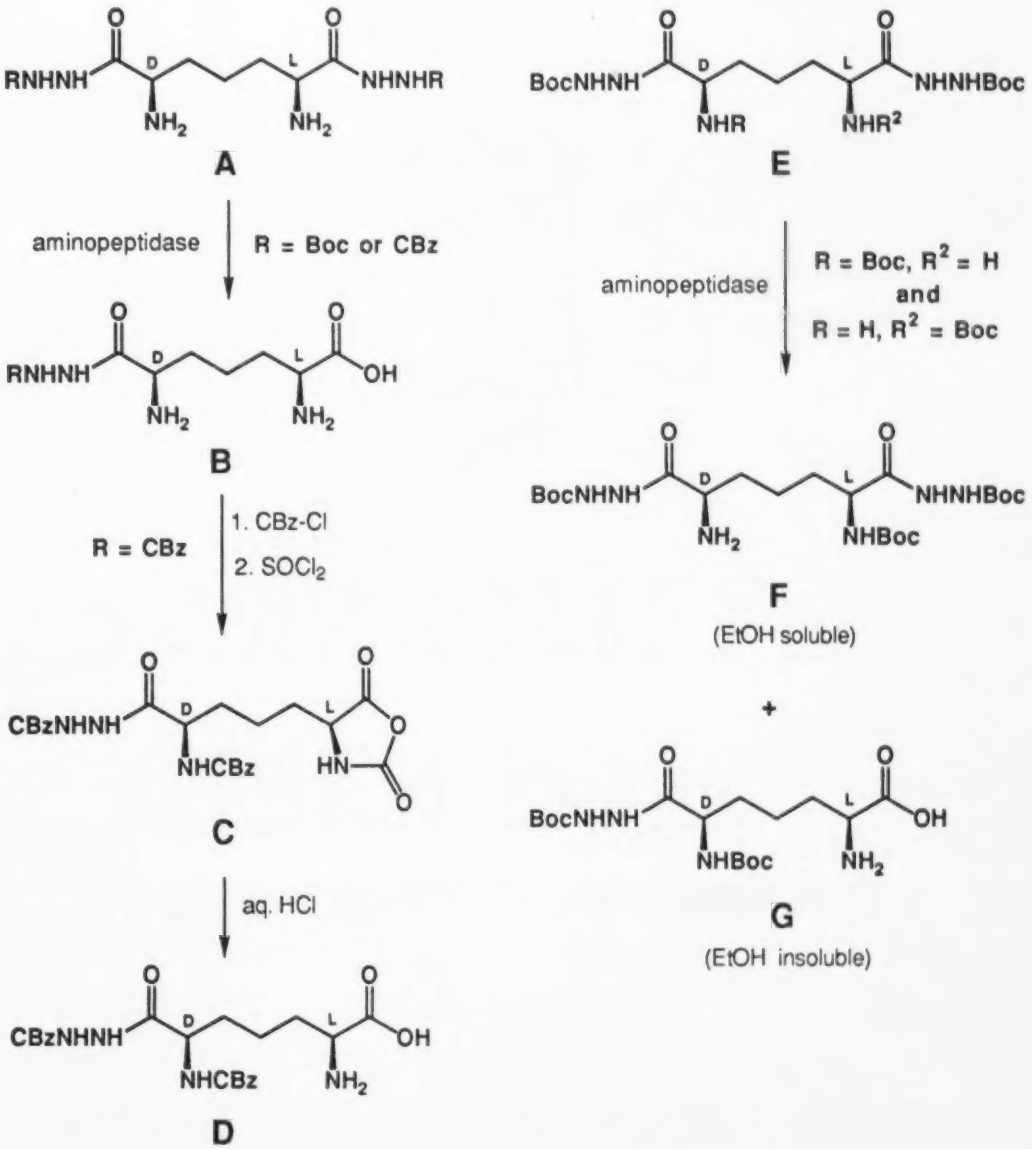
and tissues. These are presumed to originate from bacterial cell walls and may be released during macrophage digestion of bacteria.[31] These compounds show a variety of potent biological effects, although the structure-activity relationships are not yet clearly defined for all of the effects. In some cases, only the saccharide moiety or the peptide chain may be required for activity. The disaccharide pentapeptide GlcNAc-1,6-anhydro-MurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala has been identified as the causative agent of ciliated cell damage in whooping cough (*Bordetella pertussis*)[32] and gonorrhoea (*Neisseria gonorrhoeae*),[33] as well as occurring in human brain tissue as the slow-wave sleep-promoting factor, $FS_u$.[34,35] The disaccharide moiety is not neccesary for pertussis activity while the free amino and

carboxyl groups of DAP are essential. The desmuramyl peptides FK-156 (D-lactyl-L-Ala-γ-D-Glu-(L)-*meso*-DAP-(L)-Gly) isolated from *Streptomyces olivacerogriseus*[36] and the synthetic analog FK-565 (heptanoyl-γ-D-Glu-(L)-*meso*-DAP-(L)-D-Ala) have marked immunoadjuvant properties, enhancing host defences against microbial infections[37] and exhibiting strong antiviral[38] and antitumour activity.[39,40] These compounds may prove to be highly valuable as immunostimulants for people with impaired immune systems, whether due to chemotherapy or AIDS. The mammalian receptors to which this group of compounds binds have not yet been identified, however there is growing evidence that some of the effects are mediated by interleukin IL-1.[41-43]

In order to prepare compounds for the study of muramyl peptides and peptidoglycan formation, it is necessary that the different stereoisomers of DAP be available, and that *meso*-DAP be obtainable in selectively protected form so that specific reaction at either the D- or L-center becomes a matter of choice. The earliest method for obtaining the three stereoisomers separately involved enzymatic resolution by hog kidney acylase of the statistical synthetic mixture of the DAP diamides (2:1:1 of *meso*:DD:LL).[44] This method was subsequently improved by Wade *et al*[45] who initially separated the racemic pair from the *meso* isomer by fractional crystallization of their bis-carbobenzyloxy (CBz) derivatives; but the *meso* isomer was obtained with contaminating amounts of the other two isomers (see Results and Discussion section). The racemic pair has also been separated by other methods, including fractional crystallization of the diamides as their bis-benzoyl-D,D-tartrate salts (partial resolution only),[46] and by papain-catalyzed formation of the L-anilide esters of the free amines[47] or of the di-CBz derivatives.[48,49]

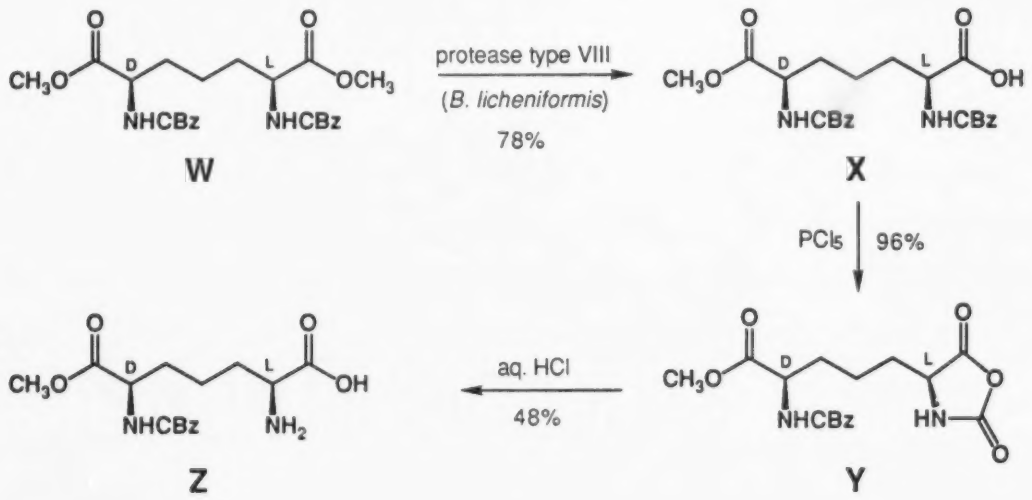
The differential protection of *meso*-DAP has been accomplished by selective enzymatic hydrolysis of various derivatives. Differential deprotection of the carboxylate groups has been achieved by selective hydrolysis of *meso*-DAP dihydrazides (**A**, **F**) to give the free carboxylate at the L-center (**B**, **H**).[50-52] Selective deprotection of one amino group has been realized by addition of carbobenzyloxy groups at both amino functionalities

of the monohydrazide **B** and cyclization of the L-center to the *N*-carboxyanhydride **C**, with subsequent cleavage to the free L-amino acid **D**.[52] Another approach to obtain mono-N-protected *meso*-DAP derivatives involves a laborious procedure which adds one *N*-carbobenzyloxy group onto the monohydrazide.[50,52] A different alternative employs enzymatic hydrolysis of the mixture of mono-*N*-Boc-protected dihydrazides[51] **E** (only the



L-center with the unprotected amino group is hydrolyzed), with subsequent separation of the two products (**F**, **G**) based on their different solubilities in ethanol.

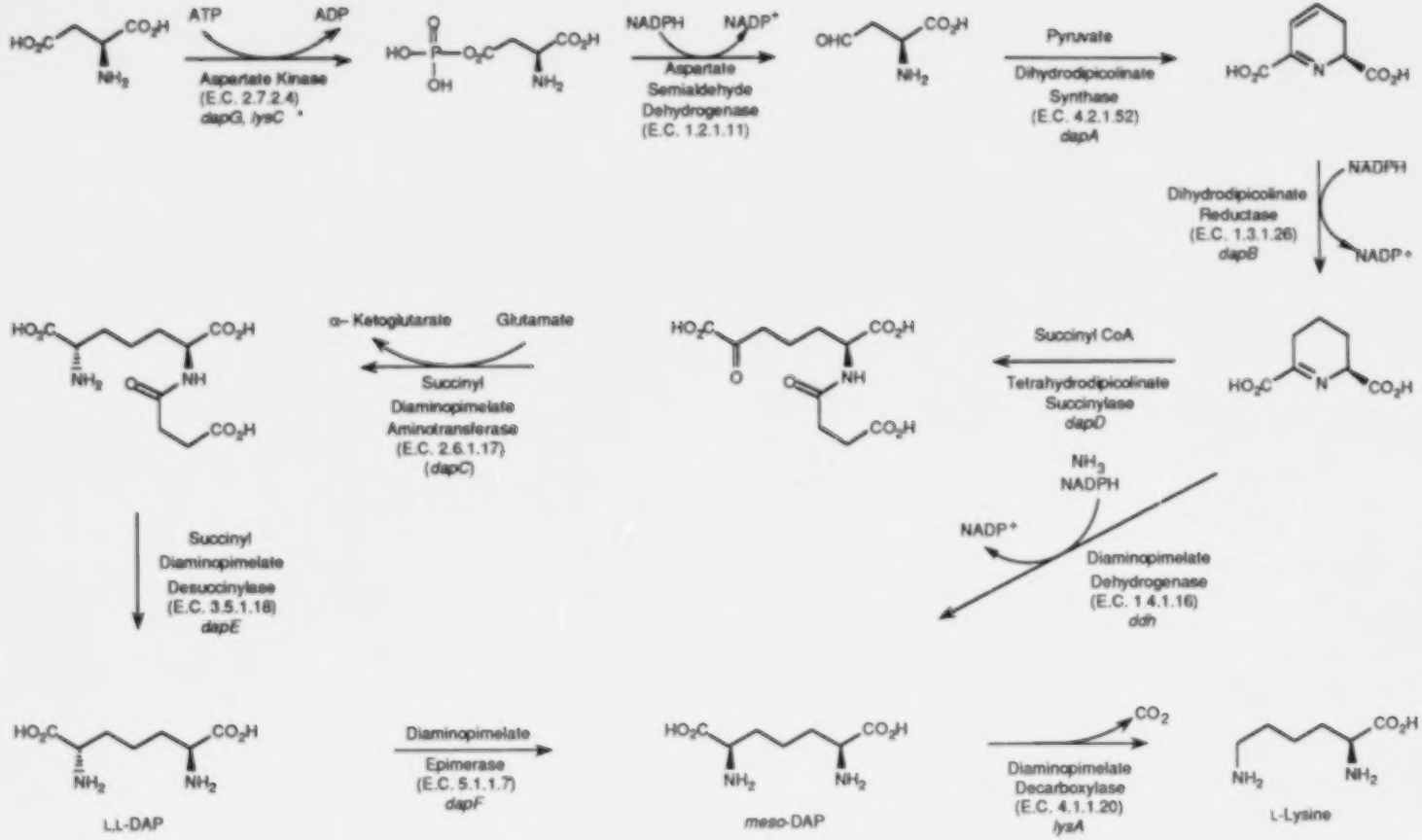
A superior route to selective enzymatic deprotection has recently been published by Kolodzieczyk *et al*[53] as part of the overall syntheses of the peptides FK-565 and FK-156. In this approach, selective hydrolysis of the L-methyl ester of di-*N*-CBz-*meso*-DAP dimethyl ester (**W**) by a commercially available protease is followed by conversion of the L-center (of **X**) to the *N*-carboxyanhydride **Y**. This is then either selectively hydrolysed in moderate yield to the free α-amino acid **Z** for subsequent N-protection (eg. Boc), or used directly in peptide coupling of the carboxyl group.




Use of DAP-containing compounds in biological testing requires that there be no significant contamination by the other stereoisomers of DAP which could cause misleading results. Therefore a stereospecific synthetic route to the DAP isomers which could also provide selective protection in the *meso* isomer is an important synthetic target. At the time our work was initiated, no stereospecific synthesis of the DAP isomers had been published. Our approaches to this challenge will be described later.

In addition to its role in bacterial peptidoglycan, DAP, in its L,L and *meso* forms, is the immediate biosynthetic precursor to lysine in bacteria[54,55] and higher plants[56-66] (Scheme **2**). L-Aspartate is converted in four steps to the key branchpoint intermediate tetrahydrodipicolinate (THDP), which in most bacteria is then N-succinylated to *N*-succinyl-2-oxo-6-L-aminopimelate by tetrahydrodipicolinate succinylase.[67,68] In many *Bacillus* species however, N-acetylation occurs instead.[69] Transamination[70,71] followed by desuccinylation[72,73] gives L,L-DAP. Epimerization to *meso*-DAP[69,74,75] is followed by decarboxylation to lysine.[76-79] In some bacteria (eg. *Bacillus sphaericus*), THDP is converted directly to *meso*-DAP by DAP-D-dehydrogenase.[80-86] Both the succinylase and dehydrogenase pathways occur simultaneously in *Corynebacterium glutamicum*,[87] whereas *Bacillus macerans* appears to have both the acetylase and dehydrogenase variants.[83]

The genes encoding the enzymes of the DAP pathway have been most thoroughly characterized for *E.coli*,[88] with all the genes being identified and cloned with the exception of the gene for the aminotransferase (*dap*C). This enzyme has never previously been purified to homogeneity or fully characterized and represents the last remaining barrier to a complete understanding of the DAP pathway in *E.coli*. The only previous isolation, by Peterkofsky and Gilvarg,[70,71] obtained the enzyme from a lysine-requiring mutant of *E.coli* in crude form with contaminating aminotransferase activity still present. After three purification steps, with ion exchange chromatography (DEAE-cellulose) as the most effective technique, they obtained an enzyme extract in 110-fold greater purity and with a specific activity of 5.33 Units/mg protein (1 Unit = 1 μmol product formed per minute). They discovered that the enzyme had a requirement for pyridoxal-5-phosphate (PLP), possessed a stability to freezing, and had optimum activity at pH 8.0 with little loss of activity until pH 5.0. The enzyme is almost completely specific for glutamate in the forward direction, while in the reverse direction there is no activity with *N*-succinyl-*meso*-DAP, L,L-DAP, *meso*-DAP, lysine, or α-CBz-lysine. Neither *N*-succinyl-*meso*-DAP nor mono-*N*-2,4-dinitrophenylhydrazinyl-L-DAP (1mM) inhibit the forward reaction.




(* The genetic markers, where known, are shown italicized.)

**Scheme 2** Lysine Biosynthesis

The enzyme is inhibited in both directions by high levels of α-ketoglutarate; however, *N*-succinyl-L,L-DAP does not show any product inhibition. Hydroxylamine causes almost complete irreversible inhibition at 50mM while ammonium sulfate causes slight inhibition. $K_{eq}$ for the forward reaction was 1.2 while the Michaelis constants ($K_m$) for the α-keto and amino acid substrates were $5.0 \times 10^{-4}$ M and $5.2 \times 10^{-3}$ M, respectively. The enzyme was assayed in the forward direction by coupling it to the desuccinylase and measuring the amount of L,L-DAP produced by a colorimetric ninhydrin method. An alternative assay coupled the forward reaction with glutamate dehydrogenase to measure the α-ketoglutarate formed, following the reduction in $\Delta A_{340}$ due to oxidation of NADH. Several groups have also assayed the aminotransferase activity in crude cell extracts.[87,88]

PLP-dependent enzymes catalyze a number of reactions of amino acids depending on which bond at the α-carbon is broken (Figure **5**). Cleavage of bond ***a*** is effected by aminotransferases and racemases, bond ***b*** by decarboxylases, and bond ***c*** by aldolases. PLP-dependent aminotransferases have been extensively studied, particularly aspartate: oxaloacetate aminotransferase.[89-96] The general mechanism is illustrated in Scheme **3**.

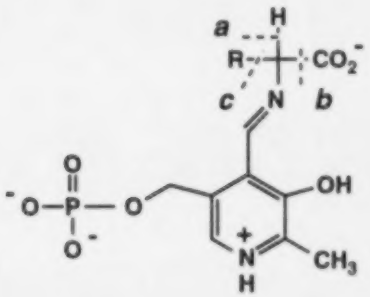
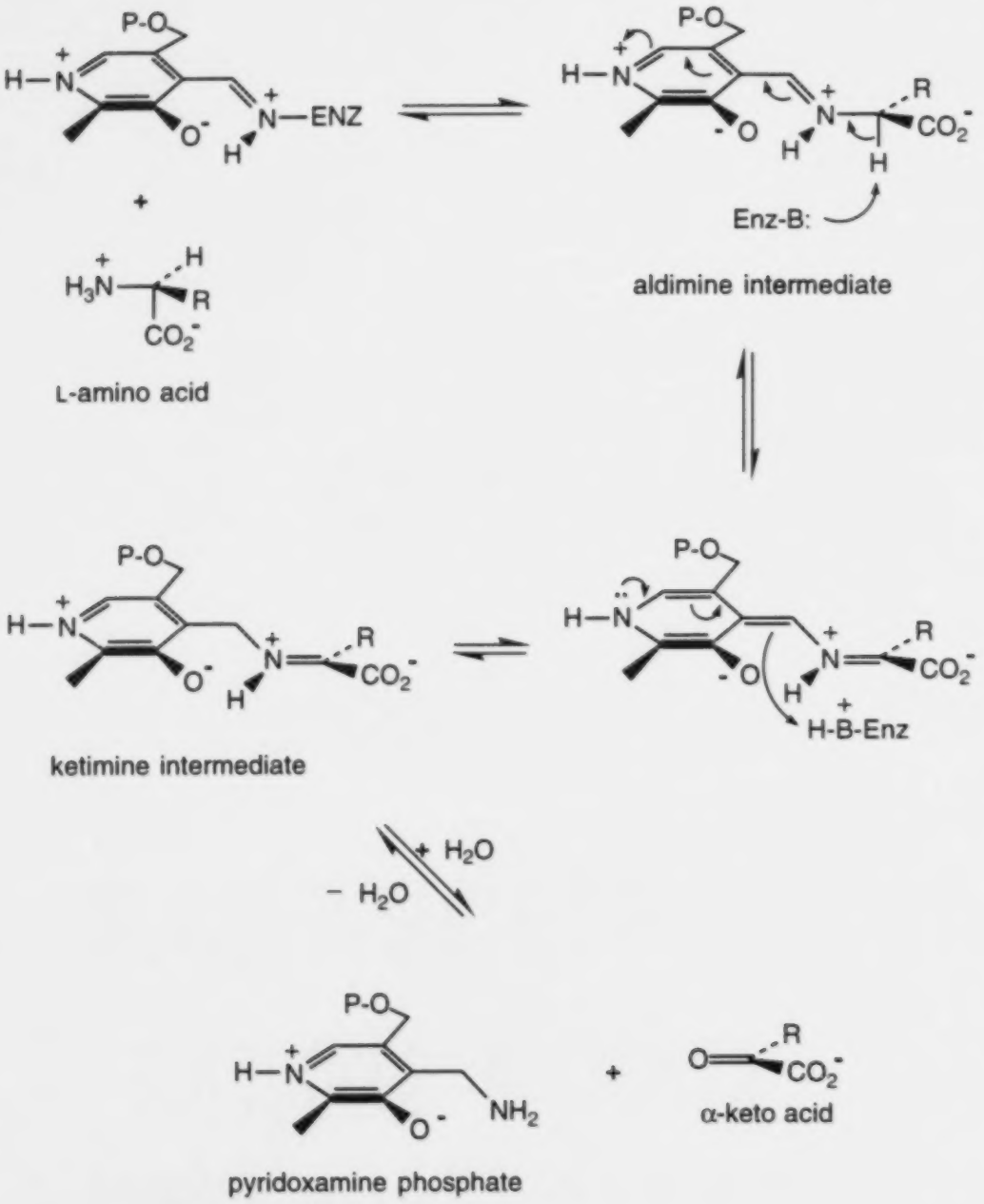



**Figure 5** Bond cleavages catalyzed by PLP-dependent enzymes




General mechanism of a PLP-dependent aminotransferase

**Scheme 3**

A number of PLP-dependent enzymes are irreversibly inhibited by *N*-hydroxy analogs of their amino acid substrates (Table **2**). These analogs form stable nitrones (Figure **6**) by transimination with PLP.[97] Hydrazino analogs of amino acids are well known as potent competitive inhibitors of PLP-dependent enzymes through formation of hydrazone intermediates (Figure **6**) which are less susceptible to hydrolysis than the regular aldimine.[98-111]

**Table 2**

*N*-Hydroxy- and *N*-amino-α-amino acid analogs as inhibitors of PLP-dependent enzymes

| Compound | PLP-Enzyme | Ref. |
|---|---|---|
| *N*-hydroxyglutamate | alanine aminotransferase, aspartate amino transferase, glutamate decarboxylase | 97 |
| *N*-aminoaspartate | aspartate aminotransferase | 98-99 |
| *N*-aminoornithine | ornithine decarboxylase, ornithine keto acid aminotransferase | 100-102 |
| *N*-aminohistidine | histidine decarboxylase | 103-105 |
| *N*-aminotryptophan | DOPA decarboxylase | 106 |
| *N*-aminophenylalanine | aromatic amino acid decarboxylase | 108 |
| *N*-aminotyrosine | aromatic amino acid decarboxylase | 108 |
| *N*-amino-DOPA | aromatic amino acid decarboxylase | 108 |
| *N*-amino-α-methyl-DOPA | aromatic amino acid decarboxylase, DOPA aminotransferase | 107 |
| *N*-aminoalanine | γ-Abu aminotransferase | 109-110 |
| *N*-amino-DAP | *meso*-DAP decarboxylase | 111 |

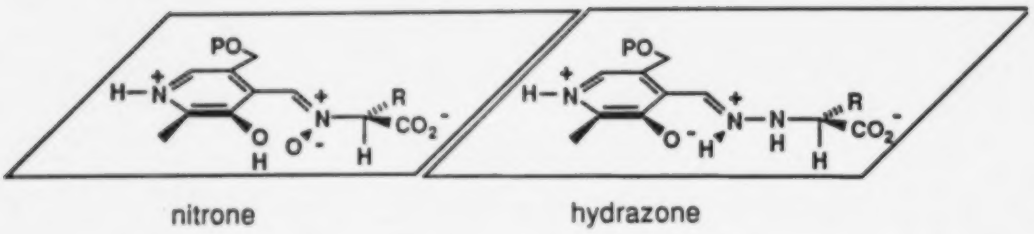
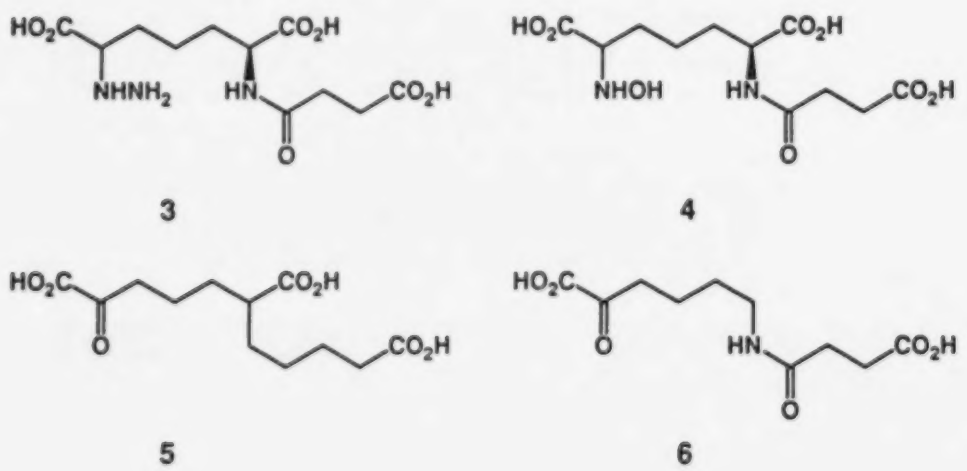



**Figure 6** Stable PLP complexes formed with amino acid analogs

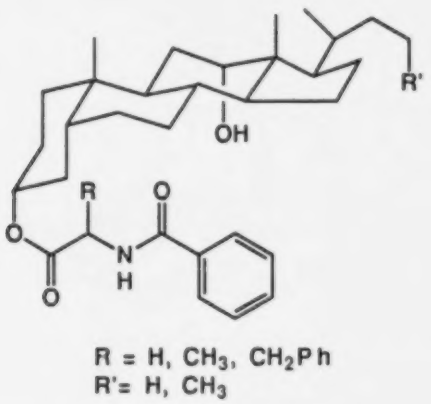
We undertook to investigate purification of the *N*-succinyl-L,L-diaminopimelate aminotransferase in order to complete the characterization of the DAP pathway in *E.coli*. This would ultimately allow identification and cloning of the corresponding structural gene. Another goal of the present project was to explore inhibition of this aminotransferase as a possible target for new antibiotics. Thus, this work also examined synthesis and testing of various analogs of the enzyme's α-amino product or α-keto substrate, including the *N*-amino **3**, *N*-hydroxy **4**, desamino **5**, and descarboxy **6** analogs.



## RESULTS AND DISCUSSION

### Stereoselective Alkylation of Chiral α-Amino Acid Esters

To investigate the use of a sterol as a chiral template for the stereoselective alkylation of amino acid esters we decided to study the *N*-benzoyl protected derivatives of glycine, alanine, and phenylalanine esterified with a side-chain-reduced deoxycholic acid derivative (Figure 7). This would allow the determination of the effects of steric bulk and pre-existing stereochemistry at the site of alkylation .

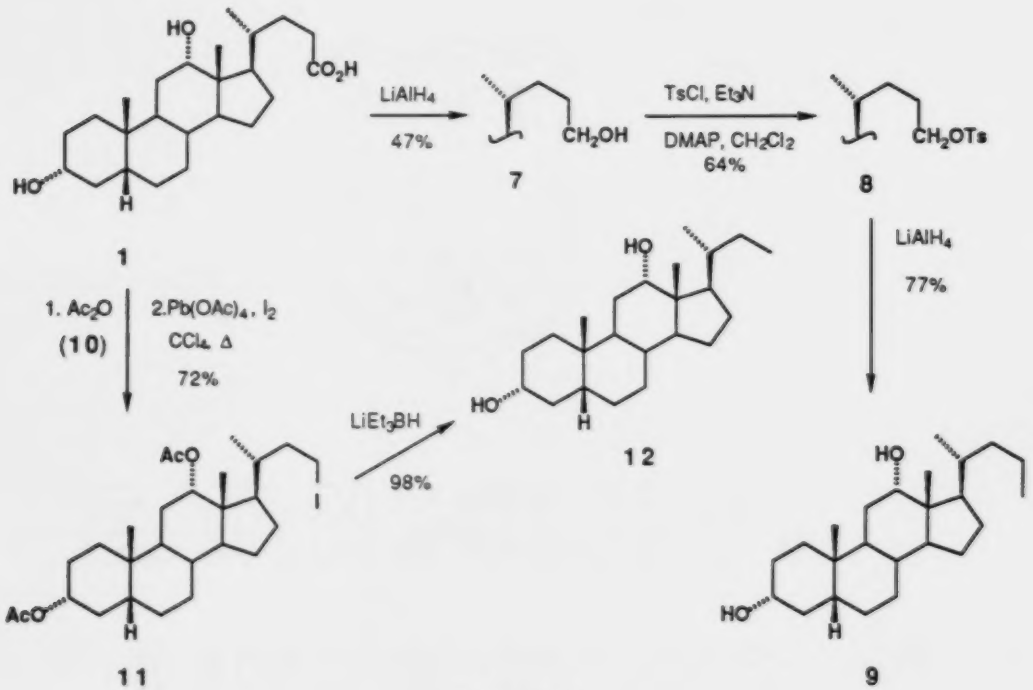



R = H, $CH_3$, $CH_2Ph$
R'= H, $CH_3$

**Figure 7** Sterol esters of *N*-benzoyl-protected amino acids

Use of deoxycholic acid (**1**) as the source of the sterol template requires the removal of the side chain carboxylate to prevent it from interfering with the complexation of the dianion intermediate generated by deprotonation of the amide and the adjacent center (as shown previously in Figure 2). This was initially achieved by $LiAlH_4$ reduction of the carboxyl group to the alcohol **7** as previously reported,[112] followed by selective tosylation of the primary hydroxyl, and subsequent reduction of **8** with $LiAlH_4$ to provide the dihydroxycholane **9**[113,114] in an overall yield of 23% (Scheme 4). In addition to being relatively low yielding, these reactions also gave complex mixtures requiring repeated

chromatographic purification. An improved method for the preparation of this compound proceeds via the diacetoxy-23-iodo-24-norcholane **11**,[112] prepared from deoxycholic acid by acetylation of the hydroxyl groups to give diacetate **10** followed by Barton's photochemical lead tetraacetate-iodine decarboxylation procedure.[115] This route provided **11** in 72% yield from deoxycholic acid. Subsequent treatment with $LiEt_3BH$ reduced the iodide and deprotected the hydroxyl groups cleanly to afford the norcholane **12** in 98% yield.




**Scheme 4**

Reagents previously used to esterify α-amino acids to the 3-hydroxyl group of sterols include dicyclohexylcarbodiimide (DCC)[116] (Figure **8**) and 1,1'-carbonyl-diimidazole (CDI);[117] however the esterification of *N*-benzoylglycine with **12** was more efficiently promoted by *N,N*-bis-(2-oxo-3-oxazolidinyl)-phosphinic chloride[118] (BOP-Cl) (Table **3**). The L- and D-*N*-benzoylalanine esters (**14** and **15**) were obtained in moderate

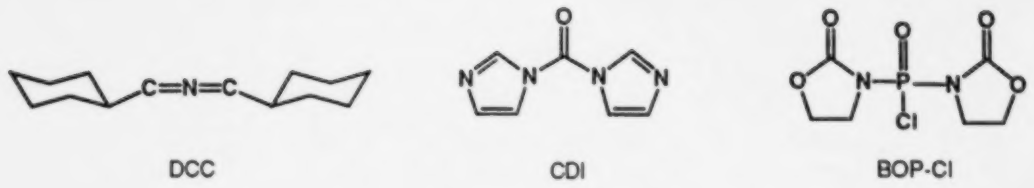
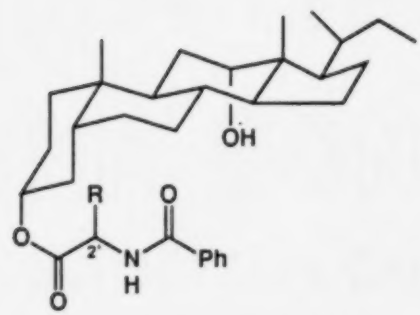



**Figure 8** Esterification reagents

yields by the allyl bromide-assisted CDI coupling of Iizuka and coworkers;[119] however, epimerization occurred during this reaction, as evidenced in the $^1$H NMR spectrum wherein the alanine methyl signal was observed as 2 doublets at δ 1.08 and 1.07 in a 52:48 ratio for **14** (by integration) and in a 60:40 ratio for **15**. Using DCC and *N*,*N*-4-dimethylamino-pyridine (DMAP) to promote the reaction also provided an epimeric mixture but in much lower yield. Esterification of *N*-benzoyl-L-phenylalanine with **12** occurred smoothly by Iizuka's procedure to give ester **16** in 62% yield, once again as an epimeric mixture at the α-centre of the amino acid. Both $^1$H NMR and TLC indicated the product is a 50:50 mixture. BOP-Cl was not an efficient reagent for this reaction.



**13**: R = H **15**: R = $CH_3$

**14**: R = $CH_3$ **16**: R = $CH_2Ph$

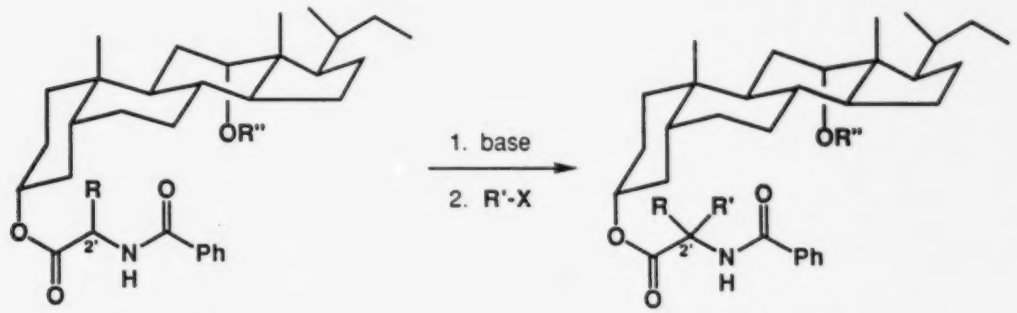
**Table 3** Esterification results

| Amino Acid | R | Reagent | Product | Yield % | Diastereomeric Ratio* |
|---|---|---|---|---|---|
| *N*-Bz-glycine | H | BOP-Cl | **13** | 74 | -- |
| *N*-Bz-L-alanine | $CH_3$ | CDI | **14** | 42 | 52:48 (R:S) |
| *N*-Bz-D-alanine | $CH_3$ | CDI | **15** | 28 | 60:40 (R:S) |
| *N*-Bz-L-phenylalanine | Bn | CDI | **16** | 62 | 50:50 |

* At the 2' center; measured by $^1$H NMR

The cause of epimerization in these reactions is not clear, however it should not affect the stereochemistry of the subsequent alkylation as deprotonation will render that center planar prior to alkylation. The general alkylation approach is illustrated in Scheme **5** and the results are summarized in Table **4**. Deprotonation of **13** with BuLi and treatment with methyl iodide in THF/HMPA at -78 °C resulted in a 70:30 mixture (by $^1$H NMR) of the D-ala:L-ala esters **17a** in moderate yield. Alternatively, using LiHMDS in THF -78 °C gave a 60:40 mixture (**17b**) in 75% yield. In order to determine whether the lack of stereoselectivity is due to poor complexation with the 12-alkoxy group, the *N*-benzoyl glycine ester **13** was converted to the 12-methoxymethyl ether (MOM ether) **18** with dimethoxymethane in order to provide a more flexible site for complexation. Alkylation with methyl iodide still afforded only a 60:40 mixture, as measured by $^1$H NMR. The presence of an α-sub-stituent might place a higher steric requirement on the direction of attack of an electrophile and conceivably lead to greater stereoselectivity in this reaction. To test this hypothesis the alanine ester **15** was alkylated with benzyl bromide. Deprotonation required the use of LDA rather than the bulkier LiHMDS. While the alkylation proceeded in high yield, very little stereoselectivity was observed (**21**, 10% d.e.). Benzylation of the 12-methoxymethyl derivative **19** gave no improvement in stereoselectivity. Conversely,

the same compound could be prepared by methylation of the phenylalanine ester **16**. This reaction proceeded in high yield but with only a moderate improvement in stereoselectivity (**23**, 30% d.e.) and the same stereochemical preference as **21**.




| | R | R" |
|---|---|---|
| 13 | H | H |
| 15 | $CH_3$ | H |
| 16 | Bn | H |
| 18 | H | MOM |
| 19 | $CH_3$ | MOM |

| | R | R" | R' |
|---|---|---|---|
| 17 | H | H | $CH_3$ |
| 20 | H | MOM | $CH_3$ |
| 21 | $CH_3$ | H | Bn |
| 22 | $CH_3$ | MOM | Bn |
| 23 | Bn | H | $CH_3$ |

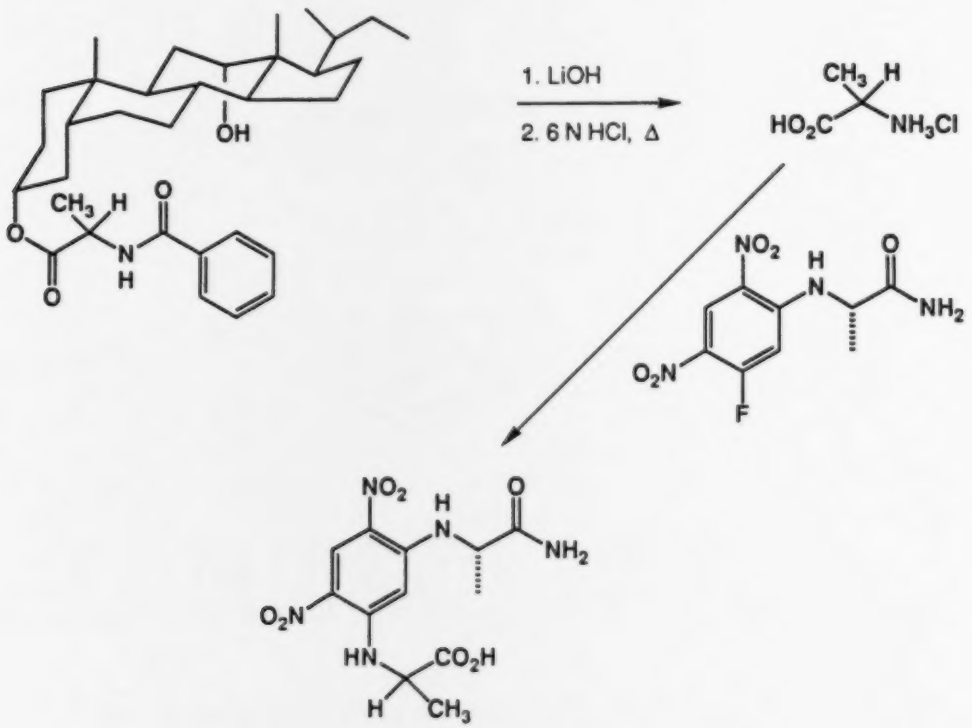
**Scheme 5**

**Table 4** Alkylation results

| Substrate | Base | R'-X | Product | Yield % | % d.e.† from: $^1$H NMR | HPLC* |
|---|---|---|---|---|---|---|
| **13** | BuLi | $CH_3I$ | **17a** | 32 | 40 | 34 (*R*) |
| **13** | LiHMDS | $CH_3I$ | **17b** | 75 | 20 | 22 (*R*) |
| **18** | LiHMDS | $CH_3I$ | **20** | 17 | 20 | -- |
| **15** | LDA | BnBr | **21** | 87 | 10 | -- |
| **19** | LDA | BnBr | **22** | 59 | 0 | -- |
| **16** | LDA | $CH_3I$ | **23** | 96 | 30 | -- |

* HPLC of Marfey derivatives  † Refers to the newly formed 2' stereogenic center

Independent confirmation of the diastereomeric ratios of **17a**, **17b**, and **15** was obtained by their hydrolysis to the free amino acids and derivatization with Marfey's reagent[120] (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) (Scheme **6**). Analysis by HPLC confirmed the previous ratios observed by $^1H$ NMR. Hydrolysis with LiOH afforded the N-protected amino acids which were subsequently cleaved to the free amino acids under strongly acidic conditions.




**Scheme 6**

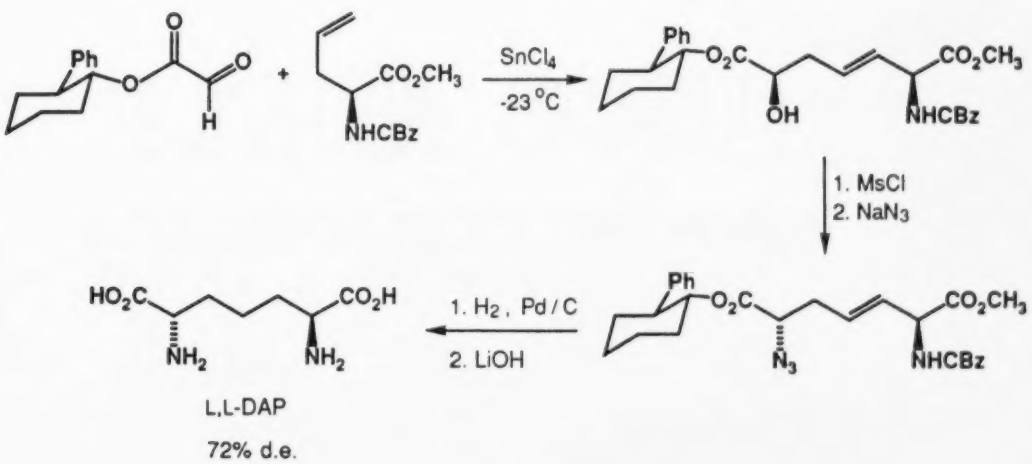
The results do not suggest that the enolate-dianion is being held underneath the sterol by metal complexation with the 12α oxygen. In the light of Prelog's study[121] on the addition of Grignard reagents to chiral α-keto esters (derived from chiral alcohols), where low but discernible stereoselectivities were observed which were dependent on the chirality

of the alcohol, it seems likely that a similar effect is responsible for the low stereoselectivities observed in these reactions. In other words, the stereoselective induction is probably due to the presence of the chiral ester group and not the formation of the desired metal complex. It is possible that aggregation of the metal enolates is also a significant factor in the chiral induction.[122] Due to the lack of chiral induction in the alkylations this approach was not pursued further.

## Synthesis of the Diaminopimelate Isomers and Their Derivatives

### 1) Stereoselective Azidation of Chiral Ester Enolates

Whitesell and co-workers have used phenylcyclohexanol as a chiral auxiliary to routinely induce diastereoselectivities of > 90% d.e. in the products of Lewis acid-catalysed ene reactions of its glyoxylate ester with various alkenes.[123-128] A stereoselective synthesis of the DAP isomers was recently achieved in the Vederas group[129] with the phenylcyclohexylglyoxylate ester in an ene reaction with *N*-CBz-L-allylglycine (Scheme 7). Conversion of the α-hydroxy group to an amine via azide displacement of its




**Scheme 7**

mesylate derivative followed by hydrogenation and ester hydrolysis affords the DAP stereoisomers in partially epimerized form.

We were interested in combining the use of the phenylcyclohexanol auxiliary with Evans' method for the stereoselective azidation of chiral enolates[9] to synthesize the DAP stereoisomers by the route shown in Figure **9**. Treatment of the bis-enolate of the chiral diester of pimelic acid (**24**) with trisyl azide (**25**) was expected to generate a single diazide isomer. Subsequent hydrogenation of the azides and hydrolysis of the esters would afford either the L,L- or D,D-DAP isomer depending on the stereochemistry of the chiral auxiliary. While the stereochemistry shown in Figure **9** is that which might intuitively be expected if the phenyl group acts to block one face of the enolate, empirical confirmation of this is required.

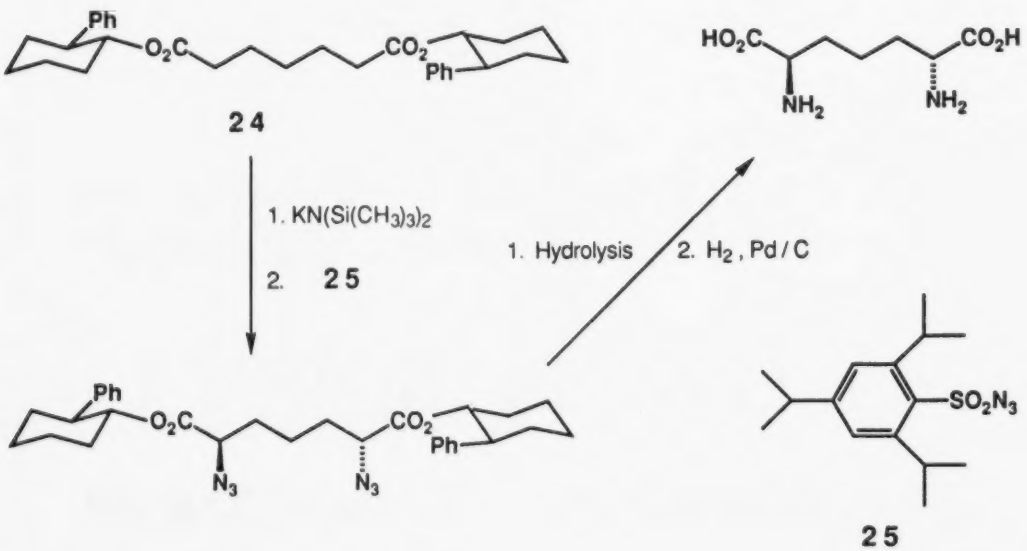
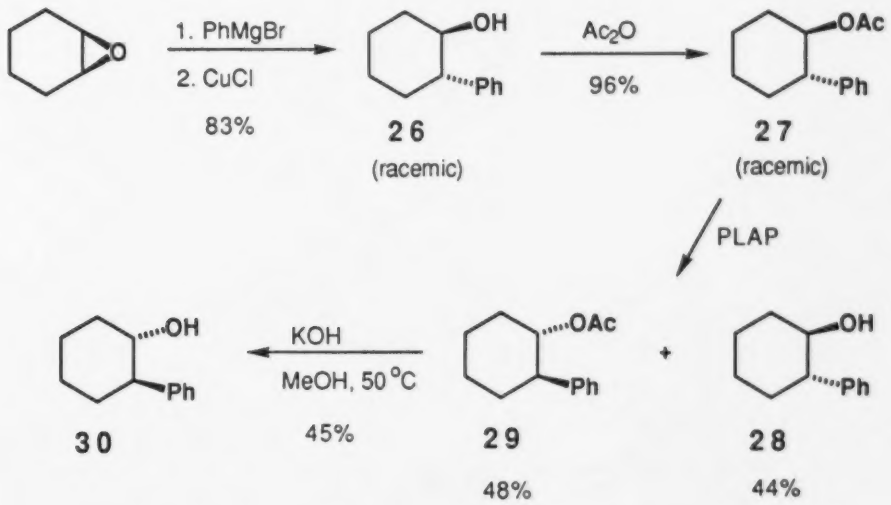



**Figure 9** Proposed stereoselective azidation route to the DAP isomers

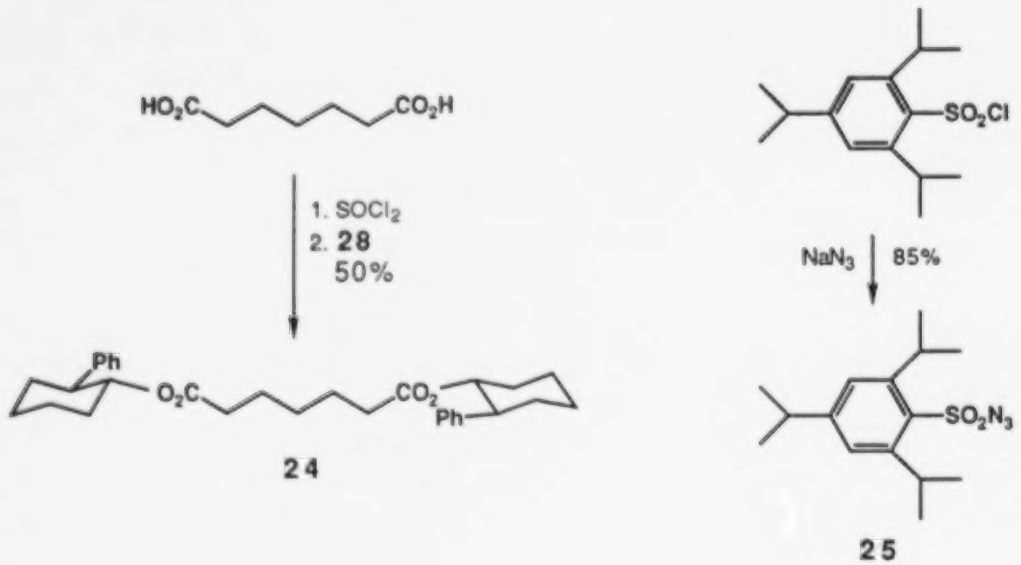
Synthesis of diester **24** required the preparation and resolution of the *trans*-2-phenylcyclohexanol enantiomers which was achieved by the method of Whitesell and Lawrence[130] (Scheme **8**). The racemic material **26** was prepared by a copper-catalyzed Grignard reaction of phenylmagnesium bromide with cyclohexene oxide. Acetylation of the racemate was followed by enzymatic resolution with pig liver acetone powder (PLAP) yielding the optically pure (-)-alcohol **28** and (+)-acetate **29**. Hydrolysis of the (+)-acetate afforded the (+)-alcohol **30** in 98.7 % e.e.




**Scheme 8**

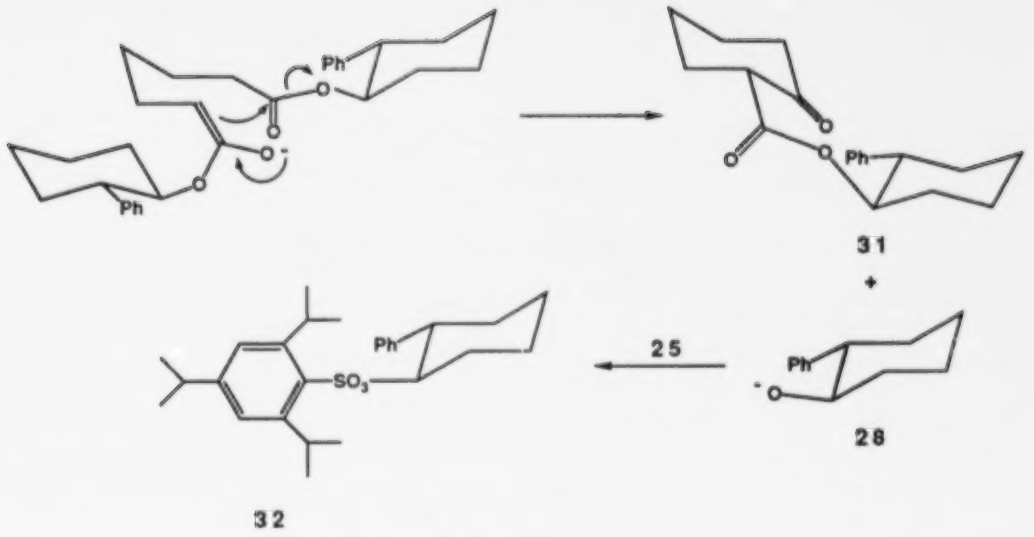
The diester **24** was obtained in moderate yield from pimelic acid by formation of the diacid chloride and reaction with the (-)-alcohol **28** in ether/pyridine (Scheme **9**). Trisyl azide (**25**) was prepared by adapting Leffler and Tsuno's method for similar arylsulfonyl azides.[131] The general method of Evans and Britton[9] was followed for the attempted azidation of **24** as illustrated in Figure **9**. Formation of the bis-enolate with KHMDS at -78 °C was followed after 0.5 h by the addition of trisyl azide, and 2 min later by the addition of AcOH. The mixture was warmed to room temperature and stirred for 12 h to allow the initially formed triazine to decompose to the azide. Work-up afforded a mixture

of four major compounds comprising starting materials (**24**, 72% recovery; **25**, 45% recovery) and two new products which were identified as the phenylcyclohexyl ester of 2-cyclohexanone carboxylic acid (**31**) and the arylsulfonate ester of phenylcyclohexanol (**32**). None of the desired product was observed.




**Scheme 9**

Compound **31** obviously results from cyclization of the mono-enolate of **24** with concomitant expulsion of the anion of phenylcyclohexanol (**28**) which then attacks trisyl azide, displacing azide and generating **32** (Scheme **10**). The fact that **31** is in equilibrium with its enol tautomer is evidenced by the signal for the enol proton at δ 12.0 in the $^{1}H$ NMR spectrum and by the OH absorption in the IR spectrum. This tautomerization causes epimerization of the stereogenic atom on the cyclohexanone ring (Figure **10**); leading to a "twinning" of many of the peaks in the $^{13}C$ NMR spectrum of the diastereomeric mixture. The obvious instability of the bis-enolate combined with the lack of any observable azide transfer ruled out this approach as a viable route to the DAP isomers.




Scheme 10

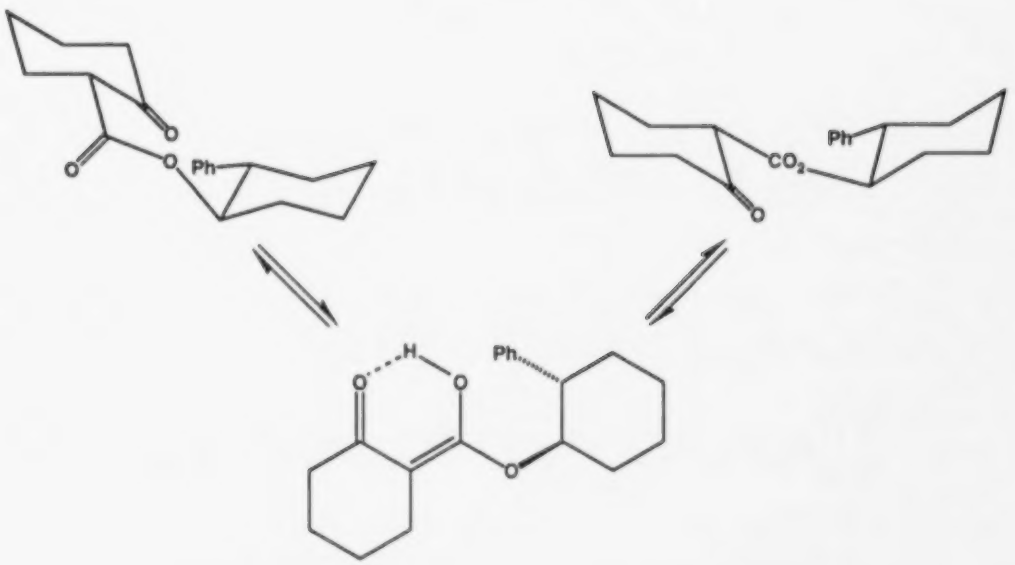
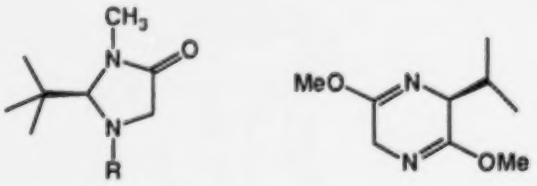



**Figure 10** Epimerization of **31** via keto-enol tautomerization

## 2) Stereoselective Alkylation of Chiral Glycine Synthons

An attempt to employ more established methods for stereoselective generation of the DAP isomers focused on the combination of the commercially available chiral glycine synthons of Seebach[132-138] (imidazolidinone) and Schöllkopf[139-141] (bis-lactim ether) (Figure **11**) . Derivatives of these compounds have been widely applied as both nucleophiles and electrophiles in syntheses of amino acids and their analogs, usually with very high stereoselectivities. Their applications have been reviewed by Williams.[2]



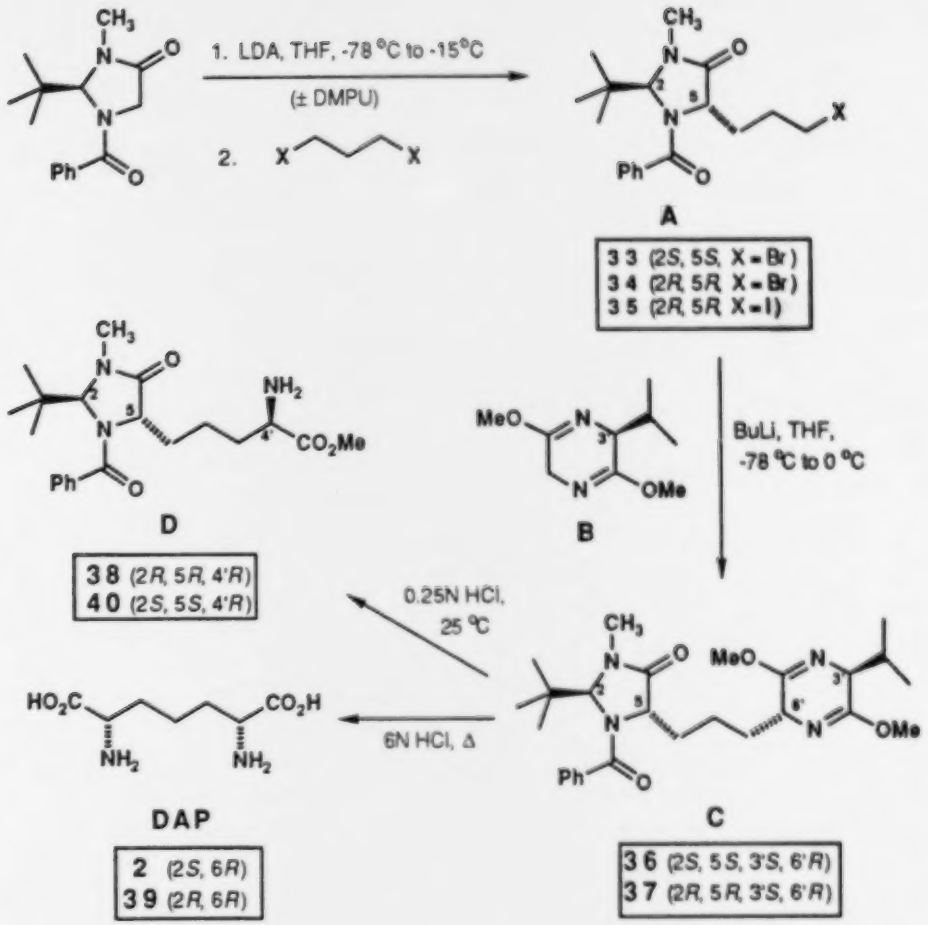

R = Bz, Boc, CBz

(Seebach) (Schöllkopf)

**Figure 11** Chiral glycine synthons of Seebach and Schöllkopf

The initial strategy for the synthesis of the DAP isomers was developed by Dr. Y. Song[142] in this research group (Scheme **11**). This involved the dialkylation of a 1,3-dihalopropane, initially with Seebach's *N*-benzoyl-imidazolidinone to give **A**, and subsequently with Schöllkopf's bis-lactim ether (**B**) to give the diprotected DAP derivative **C**. Selective hydrolysis of the more acid-labile bis-lactim ether then provides the α-amino methyl ester **D**. Alternatively, the free bis-amino acid can be obtained by strong acidic hydrolysis. This approach allows access to the three DAP isomers in selectively protected or deprotected form by appropriate choice of chirality in the reagents.

Following Dr. Song's initial studies it still remained to optimize the yields of the reactions and to determine the stereochemical purity of the final products. The results of our efforts in this area are summarized in Table **5**. Alkylation of the *N*-benzoyl-imidazolidinone with 1,3-dibromopropane is improved by the inclusion, as a co-solvent,




**Scheme 11**

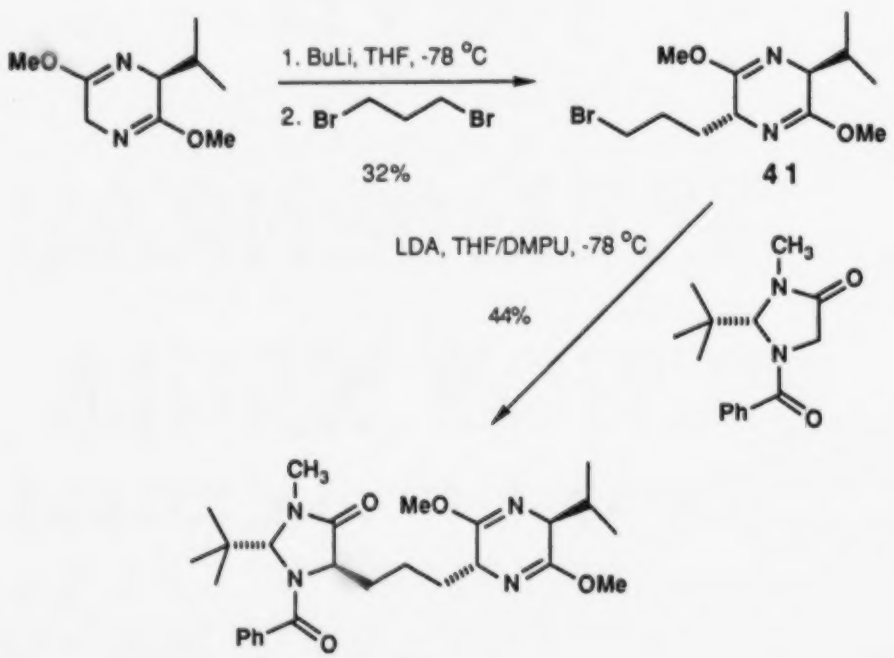
**Table 5** Results of the dialkylation route to DAP

| A | | | B | C | | D | | DAP | | |
|---|---|---|---|---|---|---|---|---|---|---|
| # | X | % | | # | % | # | % | # | % | % d.e. |
| **33*** (2*S*) | Br | 57 | 3'*S* | **36*** | 30 | **40*** | 83 | **2*** (DL) | 60 | 90 |
| **34*** (2*R*) | Br | 66 | 3'*S* | **37*** | 30 | **38*** | 86 | **39***(DD) | 68 | 46 |
| **34** (2*R*) | Br | 81 | | | | | | | | |
| **35** (2*R*) | I | 72 | 3'*S* | **37** | 48 | **38** | 67 | **39** (DD) | 56 | 75 |

* Dr. Song's results

of *N, N'*-dimethylpropyleneurea (DMPU), a less toxic alternative to hexamethylphosphoric triamide (HMPA),[143] as demonstrated by the preparation of the (2*R*)-bromopropyl-imidazolidinone (**A, 34**) in 81% yield. Preparation of the corresponding iodide (**A, 35**) by alkylation with 1,3-diiodo-propane proceeds in slightly lower yield. Subsequent alkylation with the bis-lactim ether proceeds more efficiently by displacement of the iodide rather than the bromide. Use of DMPU as a co-solvent is avoided in the second alkylation as it reduces the stereoselectivity.

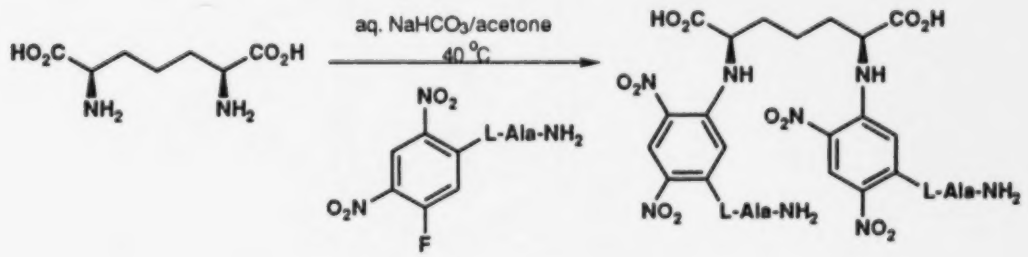
A reversal of the order of the alkylation steps was investigated to see if any improvement in the overall yield could be obtained; however, alkylation of dibromopropane with the bis lactim ether gave **41** in only 32% yield, and subsequent alkylation with the imidazolidinone afforded **37** in 44% yield. Thus, the reverse procedure gives a total yield of only 14% (Scheme **12**).




**Scheme 12**

Selective deprotection of C by hydrolysis of the bis-lactim ether proceeds smoothly in 0.25N HCl and acetone at room temperature. The epimeric monoprotected amino acid methyl esters **38** and **40** (**D**) are clearly distinguished by the signals for the proton α to the ester group in the $^1$H NMR spectra. Full deprotection of **36** and **37** (**C**) is achieved by hydrolysis in refluxing 6N HCl. Purification by cellulose chromatography gives the DAP isomers (**2, 39**) in 56-68% yield.

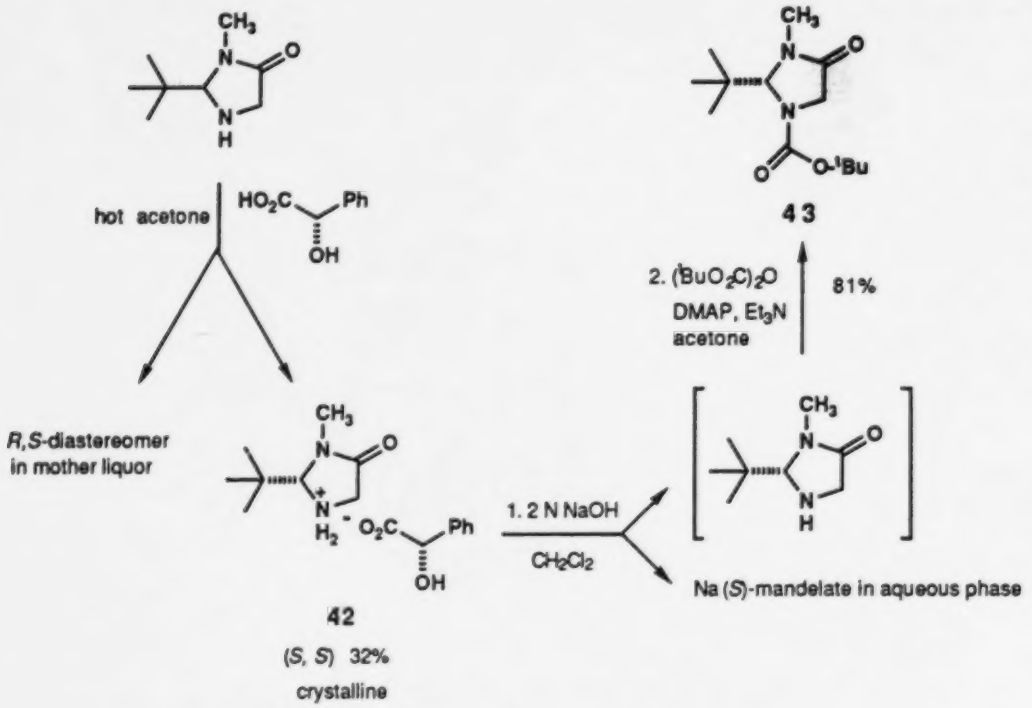
Determination of the diastereomeric purity of the DAP products was accomplished by reaction with Marfey's reagent[120] and reverse phase HPLC[144,145] of the resulting derivatives (Scheme **13**). Although the *meso*-DAP derivative (from **2**) was obtained in




**Scheme 13**

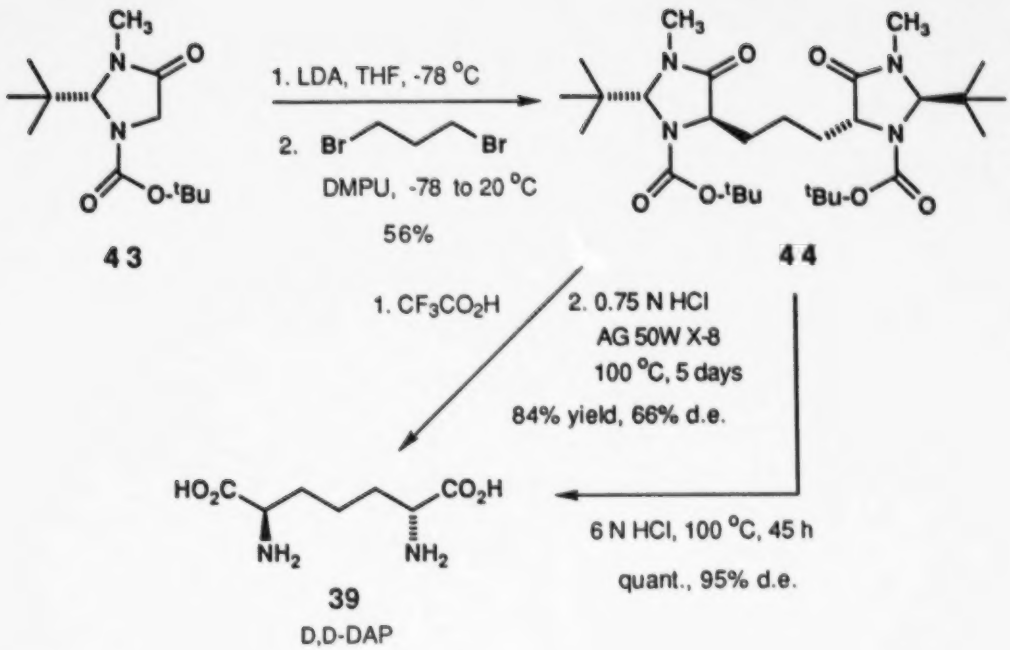
relatively high diastereomeric excess (90% d.e.), the DD-DAP derivatives (from **39**) were partially epimerized to DL-DAP (46-75% d.e.). Why **37** is more prone to epimerization during hydrolysis than **36** is not clear. Racemization during hydrolysis of the *N*-benzoyl-protected imidazolidinone group has previously been noted by Fitzi and Seebach[133] who found that this problem could be overcome by using more easily removable N-protecting groups such as CBz or Boc. Consequently, we decided to make a brief investigation of the use of the *N*-Boc-imidazolidinone (**43**) in the synthesis of the DAP isomers. The compound was not commercially available at the time and was prepared by the literature method.[146] This involved resolution of the racemic unprotected imidazolidinone (provided by Dr. Y. Pu) by fractional crystallization of the (*S*)-amine-(*S*)-(+)-mandelate salt **42**

(Scheme **14**). Conversion to the free amine and treatment with di-*tert*-butylpyrocarbonate and DMAP *in-situ* afforded the (2*R*)-*N*-Boc-imidazolidinone **43** in 81% yield and 92% optical purity.




**Scheme 14**

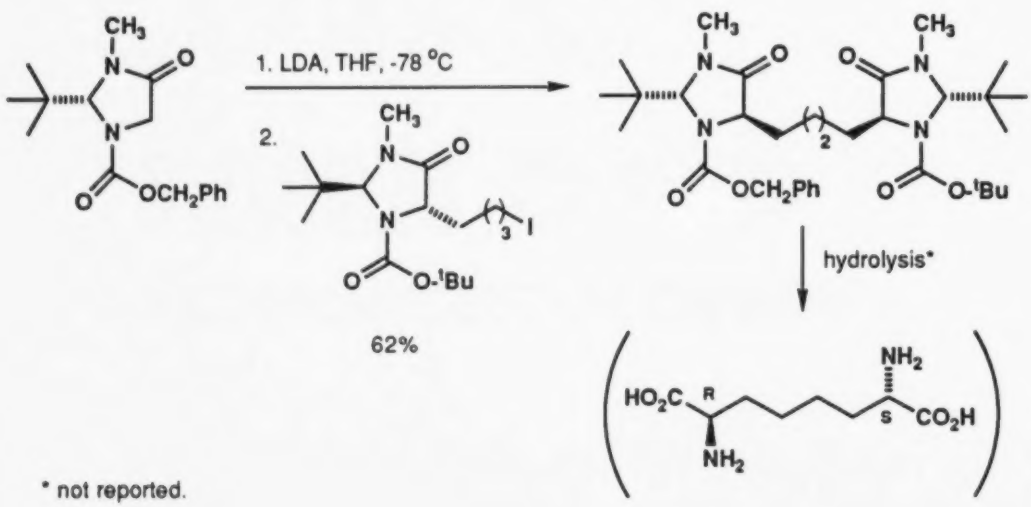
Rather than repeating the previous route to differential protection, the dialkylated product **44** was sought directly in one step, providing a route to either the LL- or DD-DAP isomers in 2 steps from **43** (Scheme **15**). Although this route was unsuccessful with the *N*-benzoyl-protected imidazolidinone,[142] Seebach and co-workers have reported the dialkylation of dihaloalkanes with the *N*-Boc-imidazolidinone.[133,134] Following the general alkylation procedure of Fitzi and Seebach,[133] the reaction of dibromopropane with the lithium enolate of **43** gave **44** in 56% yield as a white solid.




**Scheme 15**

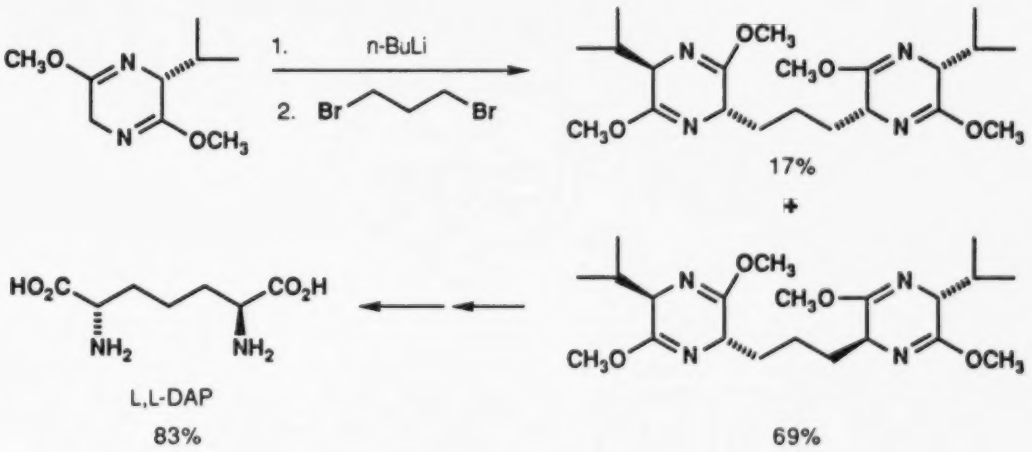
Deprotection by the literature conditions, ie. removal of the Boc groups with trifluoroacetic acid then cleavage of the imidazolidinone groups on cation exchange resin in 0.75 N HCl at 100 °C for 5 days, afforded D,D-DAP (**39**) in 84% yield but with only 66% d.e. as determined by HPLC analysis of the Marfey derivative. However, more rapid hydrolysis of **44** in 6 N HCl at 100 °C for 45 hours provided **39** in 95% d.e., suggesting that the length of exposure to acid is more important for avoiding racemization, in this case, than is the concentration of acid employed. This route provides a very simple approach to the LL- and DD-DAP isomers and avoids the difficulty of removing the L-valine methyl ester liberated in the hydrolysis of the bis-lactim ether used above. The pure *meso*-DAP isomer should also be readily available through the stepwise dialkylation of a dihalopropane with either the *N*-Boc or *N*-CBz protected imidazolidinones, both of which are reported to be cleaved to the free amino acid without racemization.[133] It may be necessary to use a differentially substituted dihalopropane (eg. chloroiodopropane) in order

to avoid dialkylation occurring in the first step, leading to L,L- or D,D-DAP isomers. The less reactive chloride could be readily replaced, if neccessary, by iodide in a Finkelstein reaction[134] prior to the second alkylation. The feasibility of this route has been demonstrated by Seebach *et al*[134] who made the differentially protected homolog in an overall 62% yield for both alkylation steps without proceeding on to the free diamino-suberic acid (Scheme **16**). The possibility of selective deprotection of such a derivative to allow subsequent reaction specifically at either the D or L center is not obvious. Even if the N-protecting groups can be selectively removed, it is questionable whether either group would survive the relatively harsh acidic conditions needed for cleavage of the imidazolidinone ring. Substitution of the *N*-CBz-imidazolidinone for the *N*-benzoyl derivative in the previous DAP synthesis (Scheme **11**) should allow selective protection of a *meso*-DAP derivative by cleavage of the bis lactim ether in the presence of the CBz group. Manipulations of the mono-protected compound (eg peptide formation) should survive the subsequent hydrolysis of the *N*-CBz-imidazolidinone, due to the milder conditions required compared to the *N*-benzoyl derivative.




**Scheme 16**

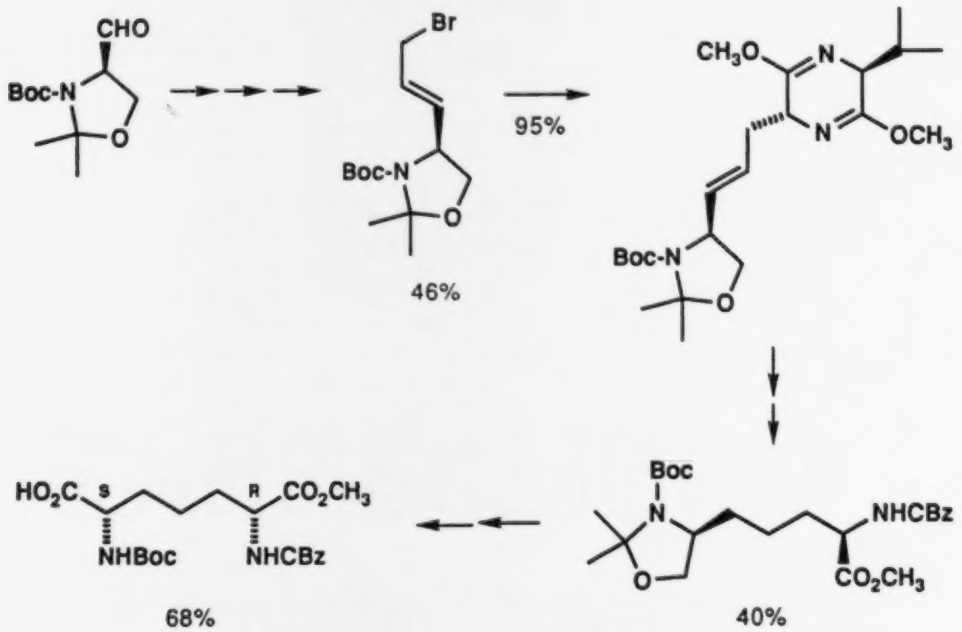
Since this work was completed, three separate syntheses of DAP diastereomers have appeared in the literature. The first method by Bold *et al*[147] utilizes Schöllkopf's bis-lactim ether to prepare L,L-DAP in a route analogous to the imidazolidinone dialkylation described above (Scheme **17**). They obtain a mixture of two diastereomers which are separated chromatographically. Deprotection to either the dimethyl ester or the free L,L- or D,D-DAP proceeds in high yield. This is currently the best route to these isomers due to its brevity and high efficiency. The bis-lactim ethers are also considerably cheaper than the Boc- or CBz-protected imidazolidinones.




**Scheme 17**

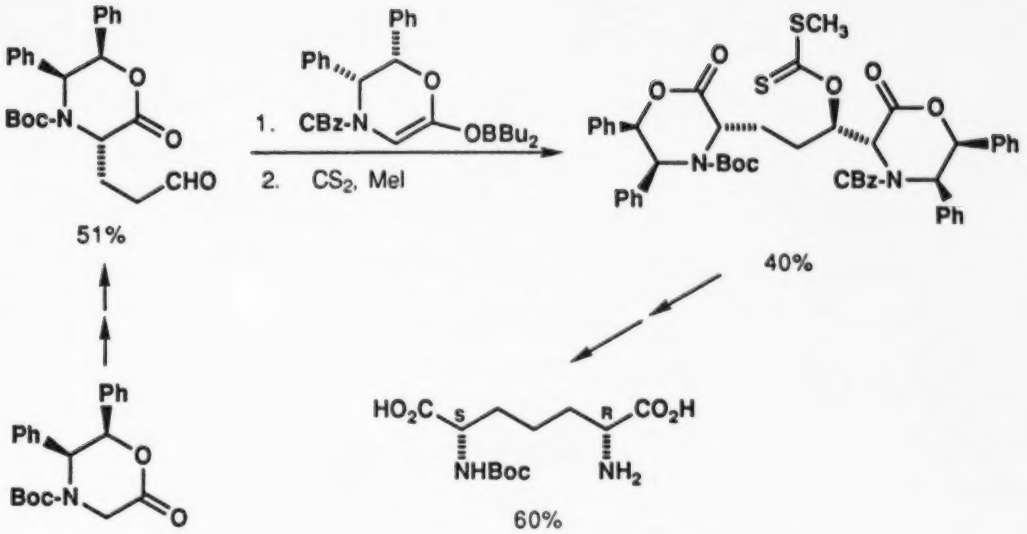
The second method, by Jurgens,[148] describes the synthesis of differentially protected *meso*-DAP through combined use of the D-serine-derived Garner oxazolidine[147,149] and Schöllkopf's bis-lactim ether (Scheme **18**). Selective cleavage of the alkylated bis-lactim ether allows differentiation of the two ends. The synthesis proceeds in nine steps from the oxazolidine (which must also be prepared) in an overall yield of 12%.

In the last method, Williams and Yuan[150] describe the preparation of all the DAP isomers, including a mono-N-protected *meso*-DAP, through the enol-borane aldol coupling of differentially N-protected diphenyloxazinone templates (Scheme **19**) with subsequent removal of the hydroxyl group by radical reduction of the methyl xanthate ester. The mono-*N*-Boc-*meso*-DAP is obtained in 6 steps and 12% overall yield. An alternative alkylation method utilizing Baldwin's modification of their procedure[151] fails to realize a significant yield.



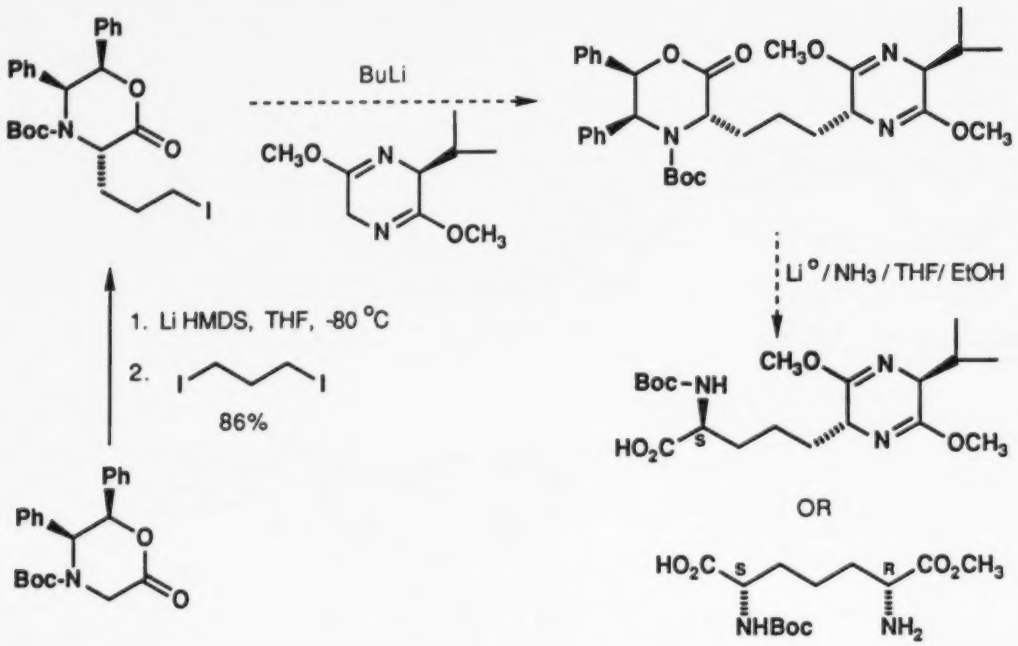

**Scheme 18**

Neither of these routes to selectively protected *meso*-DAP is ideal. Williams' route is shorter but low yielding. Jurgens' method does, however, provide a higher level of selective protection with the same overall yield. A dialkylation route to *meso*-DAP is therefore still a desirable target. While our previously described dialkylation route provides *meso*-DAP in 90% d.e., substitution of the *N*-benzoyl imidazolidinone by either




**Scheme 19**

the *N*-Boc or *N*-CBz derivatives, with their milder hydrolysis requirements, could eliminate the epimerization occurring in this step. Alternatively, repeated stepwise alkylation with either of the latter compounds could also furnish *meso*-DAP, providing selective protection is not required. Differentially protected *meso*-DAP represents more of a challenge. The deprotection method of Kolodzieczyk *et al*[53] (described in the Introduction), through selective enzymatic ester hydrolysis of di-CBz-*meso*-DAP dimethyl ester, represents one possible route, providing that a source of the pure *meso*-DAP isomer is available. In terms of a synthetic approach, selective protection by dialkylation with differentially protected imidazolidinones seems unrealistic due to the harsh conditions required for hydrolysis. Stepwise dialkylation with Williams' oxazoline does not proceed in high yield after the first alkylation, while reaction of Schöllkopf's bis lactim ether with dibromopropane leads to initial dialkylation, or cyclization if the more reactive diiodide is used.[152] As the alkylation properties of these two templates seem to be complementary, their combined use in a synthesis of selectively protected *meso*-DAP would appear to be quite promising (Scheme **20**).



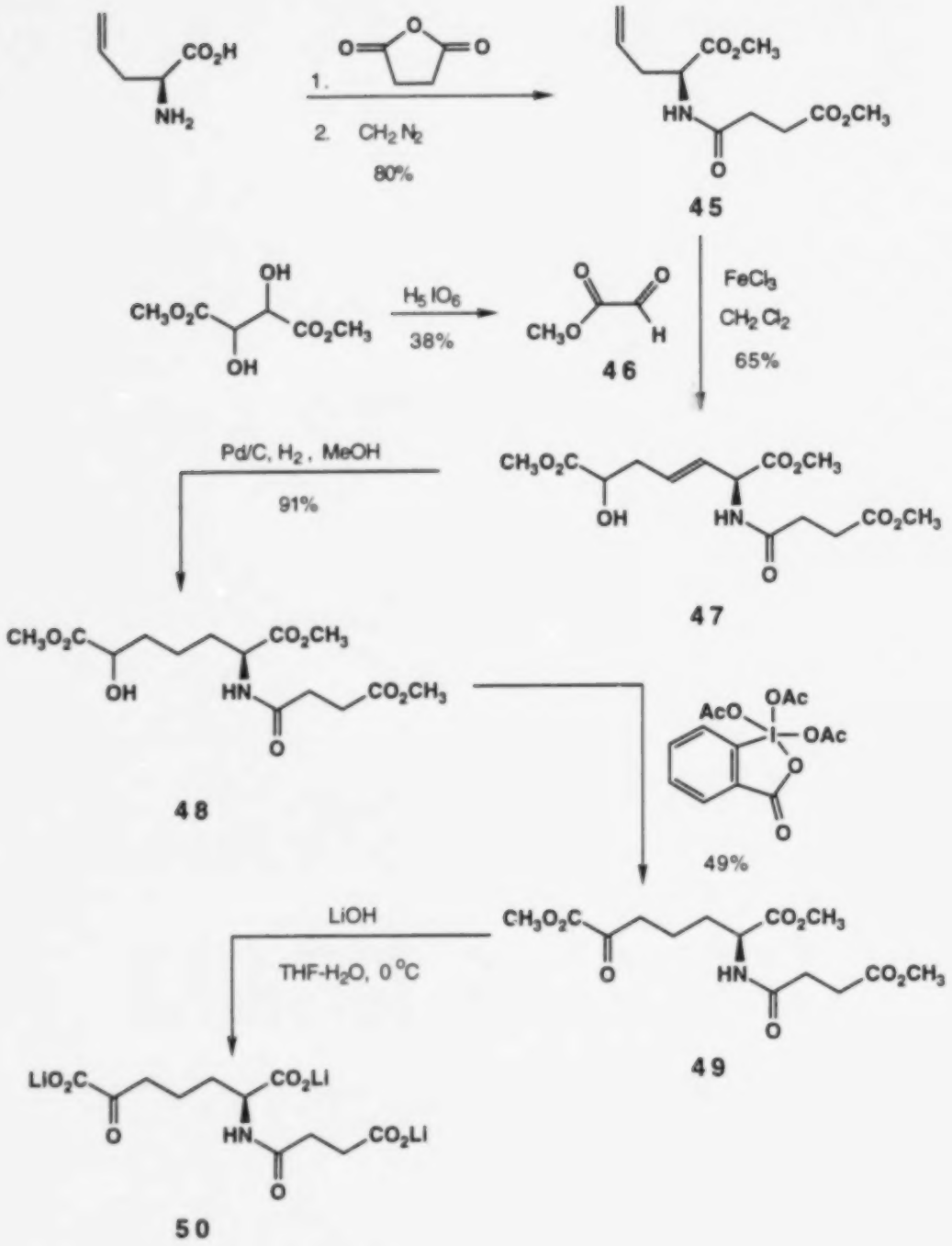

**Scheme 20**

The alkylation of the *N*-Boc protected oxazinone with diiodopropane has been reported by Williams and Im[153] to proceed in 86% yield. Alkylation of the bis lactim ether should be feasible, and subsequent deprotection of the oxazinone by dissolving metal reduction[153] to the *N*-Boc amino acid would provide the desired selective protection providing the bis lactim ether was retained. Alternatively, cleavage of both groups would provide the mono-*N*-Boc-mono-methyl ester which could then be differentially N-protected.

## Studies on *N*-Succinyl Diaminopimelate Aminotransferase

### Preparation of the Enzyme's α-Keto Substrate

The α-keto substrate, (6*S*)-6-*N*-succinylamino-2-oxo-heptan-1,7-dioate (**50**), is required for the enzyme assay. Previously this compound had only been obtained by isolation from natural sources or by enzymatic conversion from LL-DAP (obtained from natural sources) in 5% yield.[67] A synthesis of the compound was devised by Dr. Lister Lam following the route to unsaturated pimelic acid derivatives developed by Agouridas *et al.*[154,155] The key step of this synthesis (Scheme **21**) is the Lewis acid-catalyzed ene reaction between *N*-succinyl-L-allylglycine dimethyl ester (**45**) and methyl glyoxylate (**46**) which gives the unsaturated α-hydroxy triester **47**. Methyl glyoxylate was obtained by the oxidation of dimethyl tartrate by periodic acid.[156] Compound **45** was prepared by Dr. Russell Cox by treatment of L-allylglycine with succinic anhydride and subsequent methylation with diazomethane. The ene reaction affords **47** in moderate yield and subsequent hydrogenation of the double bond gives **48** in high yield. Oxidation to the α-keto ester **49** was originally achieved in low yield with a mixture of $KMnO_4$ and $CuSO_4$.[157] However, it was subsequently found that much better results could be obtained with the Dess-Martin periodinane.[158-161] Compound **49** was obtained in 49% yield by this method, but subsequently yields of up to 79% have been obtained. Hydrolysis to the free triacid with LiOH in THF/$H_2O$ (1:3) at 0 °C proceeds smoothly, but the work-up and purification of the product proved to be difficult. The original work-up conditions consisted of acidification of the hydrolysate with 1 N HCl and extraction into EtOAc. The combination of a low partition ratio and acidic conditions resulted in a low recovery of the product. All attempts at purification of the triacid, by HPLC, RP-MPLC, hydrophobic interaction chromatography, or ion exchange chromatography led to decomposition. Recrystallization also proved unsuccessful, in accord with previous work.[67] The compound appears to be particularly sensitive to acidic conditions, probably due to the succinamide group which


CO2H
NH2
1.
2. CH2N2
80%
CO2CH3
HN
CO2CH3
O
45
OH
CH3O2C
CO2CH3
OH
H5IO6
38%
CH3O
H
46
FeCl3
CH2Cl2
65%
Pd/C, H2, MeOH
91%
47
48
AcO
OAc
OAc
49%
LiOH
THF-H2O, 0 °C
49
LiO2C
CO2Li
50

**Scheme 21**

may cyclize to a succinimide or eliminate succinic anhydride under acidic conditions. It has previously been noted that some α-ketoacids are more stable as their carboxylate salts, which are less prone to decarboxylation and polymerization.[162] Thus, the product **50** was retained as the tri-lithium salt after hydrolysis with exactly three equivalents of LiOH. Characterization of the product by $^{1}H$ and $^{13}C$ NMR gave results consistent with the desired product. However, the salt proved no more amenable to crystallization than the free acid and consequently failed to give accurate combustion analysis, probably due to its hygroscopic nature. A strong $M^{+}$ peak was observed in the FAB mass spectrum. Disappearance of the $^{1}H$ NMR signals for the protons α to the keto group on standing in $D_2O$ is attributed to exchange with $^{2}H$ through keto-enol tautomerization (Figure **12**). This is supported by the appearance of $^{2}H$ coupling to the α-carbon in the $^{13}C$ NMR spectrum.

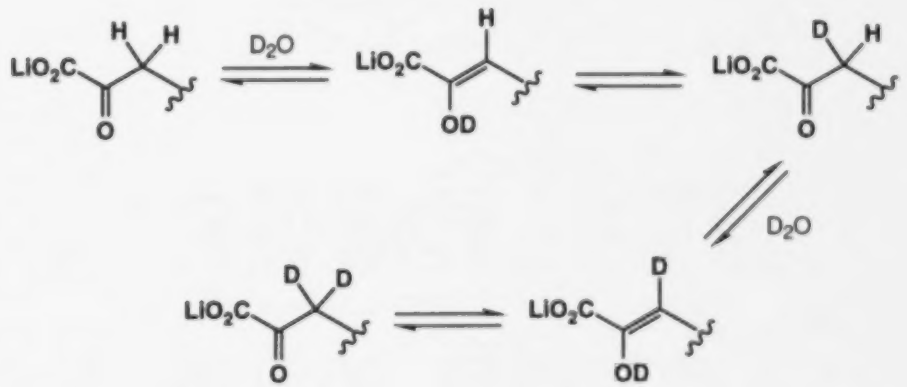


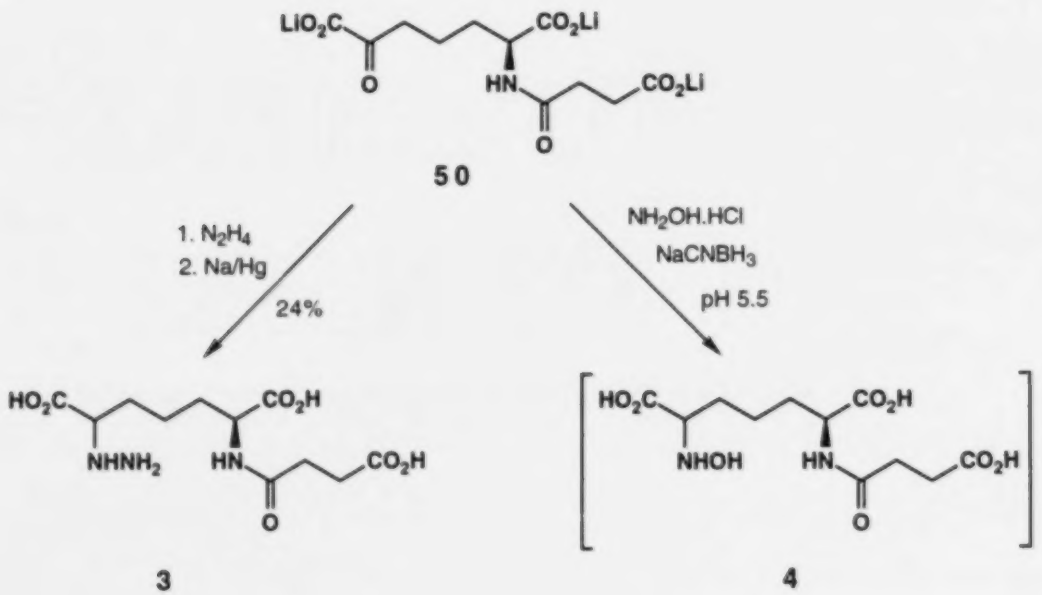

**Figure 12** Mechanism of $^{2}H$ incorporation into **50**.

### 2-*N*-Amino- and 2-*N*-Hydroxy-6-*N*-L-succinyldiaminopimelate

Preparation of the *N*-amino **3** and *N*-hydroxy **4** analogs of *N*-succinyl DAP, the product of the enzyme reaction, was attempted from the α-keto enzyme substrate **50**. The conversion of α-keto acids to α-hydrazino acids by reaction with hydrazine to form the hydrazone

followed by reduction with sodium amalgam has previously been used to prepare a variety of these compounds.[106,163-165] The 2-*N*-amino-*N*-succinyl DAP (**3**) was prepared in this fashion in 24% yield from **50** (Scheme **22**).

The *N*-hydroxy-*N*-succinyl DAP (**4**) proved to be more difficult to obtain as a stable material. The preparation of α-hydroxyamino acids from the corresponding α-keto acids has been accomplished by oxime formation with hydroxylamine and reduction with excess $NaCNBH_3$ *in situ*.[97,166,167] The remaining $NaCNBH_3$ is decomposed at acidic pH and the product then obtained by cation exchange chromatography. In this case the acidic conditions may lead to decomposition or elimination of the succinyl moiety. Furthermore, *N*-hydroxyamino acids are inherently unstable and have been reported to disproportionate in aqueous solution to the amino acid and the oxime, which can further decarboxylate to the corresponding nitrile[168] or, if an aromatic substituent is present, to the aldoxime.[169,170]

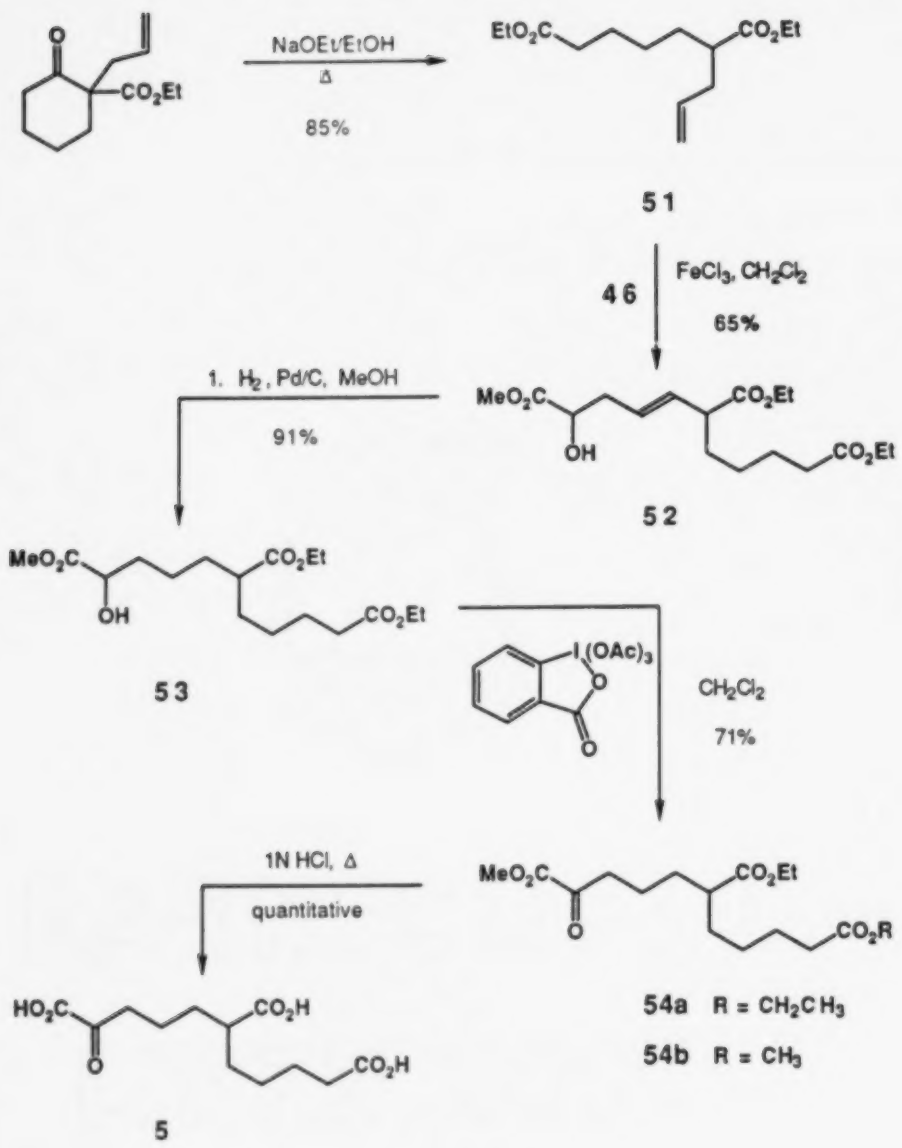



**Scheme 22**

The physical properties, natural occurrence and syntheses of *N*-hydroxyamino acids have been reviewed.[171] *N*-Hydroxy-amino acids are reportedly only stable when stored at low temperature as solids, preferably as the mineral salts (eg. hydrochloride), a condition which once again appears to be incompatible with the *N*-succinyl group. Not surprisingly, the product obtained from the reaction of **50** under the above conditions failed to give satisfactory characterization data. The material obtained from the cation exchange column did give a positive result (black color) when spotted on a silica TLC plate and sprayed with $AgNO_3$ solution. This reducing ability of α-hydroxyamino acids is due to their oxidation to the oxime. When a sample was run on a silica TLC plate in n-BuOH:AcOH:$H_2O$, however, no reducing spot was observed with $AgNO_3$, and only a single ninhydrin-positive spot appeared which had an $R_f$ corresponding to that of an authentic sample of *N*-succinyl DAP. In addition, **4** should be a potent inhibitor of the aminotransferase, however, when the product was tested it showed only weak activity, also suggestive of prior decomposition. In conclusion, it would appear that the N-hydroxy-amine analog **4** is inherently unstable due to the sensitivity of the succinamide to the acidic conditions that the hydroxyamino group requires for stability.

**Desamino and Descarboxy α-Keto Substrate Analogs**

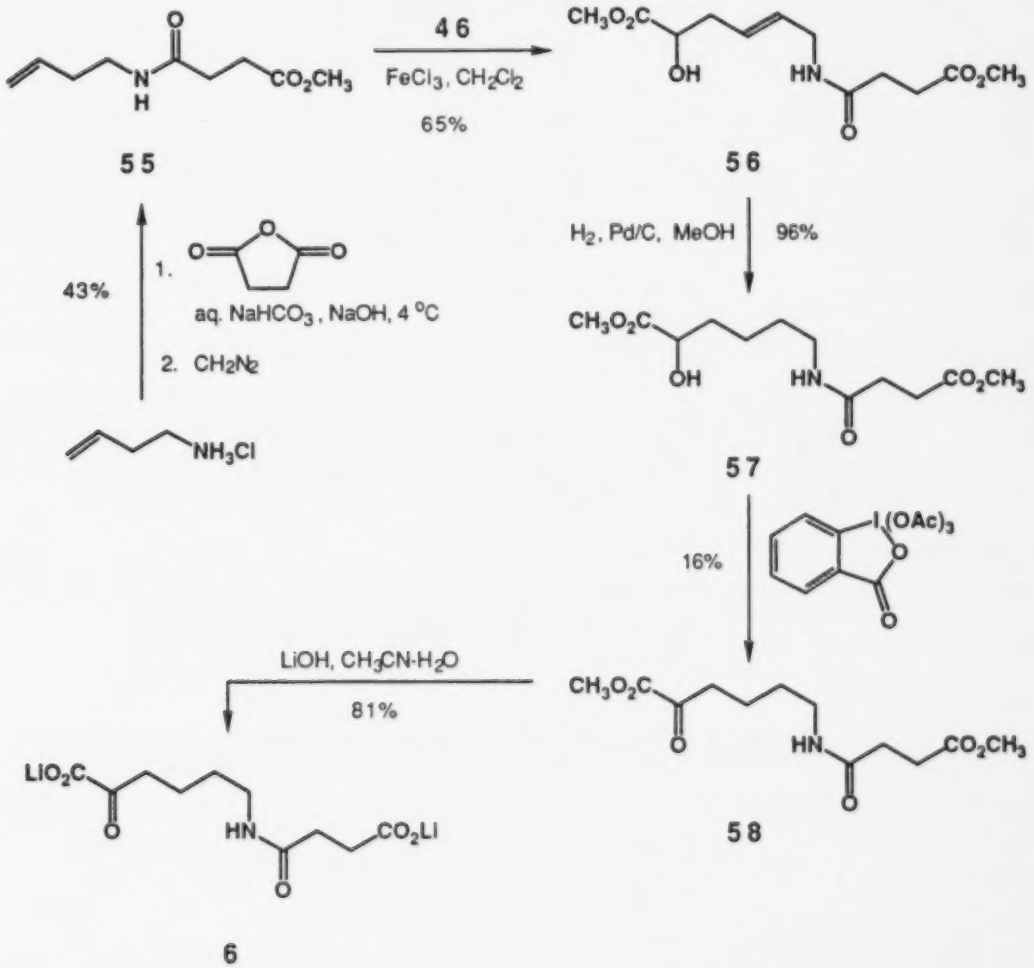
The initial syntheses of the desamino **5** and descarboxy **6** α-keto substrate analogs were designed and partly completed by Dr. Lister Lam of our group. The final steps of the syntheses were investigated by Dr. Russell Cox. The synthetic route to the desamino analog **5** is shown in Scheme **23**. Ethyl 1-allyl-2-cyclohexanonecarboxylate[172] was treated with NaOEt in an adaptation of the literature procedure[173] to afford the ring-opened unsaturated diester **51**. Reaction with methyl glyoxylate (**46**) in the $FeCl_3$-catalyzed ene reaction provided the α-hydroxy-unsaturated tri-ester **52**. During hydrogenation of the double bond some transesterification of the 6-carboethoxy group with the solvent occurred, giving a mixture of diethyl and dimethyl esters **53** which were carried on as a mixture.




**Scheme 23**

Oxidation with the Dess-Martin periodinane afforded the α-keto esters **54a** and **54b**. Hydrolysis with LiOH did not fully deprotect the 6-carboethoxy group, however, in the absence of the succinamide functionality, acid hydrolysis was feasible and yielded the triacid **5** quantitatively.

The preparation of the descarboxy analog **6** was also approached via an ene reaction with methyl glyoxylate (Scheme **24**). The alkene **55** is prepared from 3-butenylamine hydro-chloride by treatment with succinic anhydride to give the succinamide, followed by esterification with diazomethane. Hydrogenation of the ene-reaction adduct **56**, then oxidation of **57** gives a low yield of the α-keto diester **58**. Basic hydrolysis provides the descarboxy compound **6** as the di-lithium salt in 81% yield.




**Scheme 24**

**Activity and Protein Assays**

In previous studies of this aminotransferase, the activity had been determined using several different assay systems. In the original isolation from *E. coli* by Peterkofsky and Gilvarg[70,71] the activity was measured with a discontinuous coupled assay wherein the *N*-succinyl-DAP produced by the aminotransferase was converted by *N*-succinyl-DAP desuccinylase to L,L-DAP, which was then quantified colorimetrically by reaction with ninhydrin. An alternative coupled assay wherein the α-ketoglutarate produced by the aminotransferase was recycled to glutamate by glutamate dehydrogenase[174] with concomitant stoichiometric oxidation of NADH, was followed spectrophotometrically by the reduction of absorbance at 340 nm ($\Delta A_{340}$) due to the loss of the NADH chromophore (Figure 13). More recently the enzyme activity for the reverse reaction has been measured by discontinous assays of crude cell extracts from *Corynebacterium glutamicum*[87] and *E.coli*[88] by following the disappearance of the *N*-succinyl DAP substrate. For our study the coupled assay of Peterkofsky and Gilvarg was used with minor modifications. The buffer conditions, pH, and temperature were left unchanged, but the glutamate dehydrogenase chosen for the assay was the NADPH-specific isozyme from yeast (Sigma,

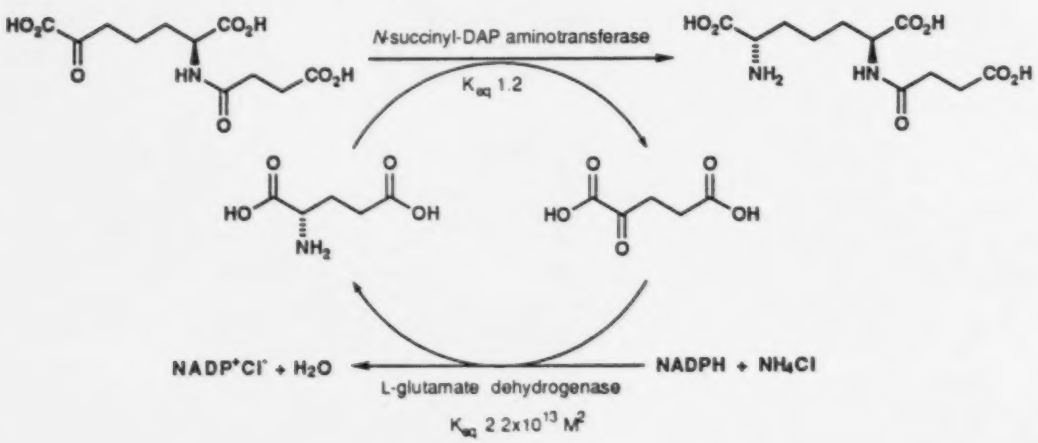



**Figure 13** Coupled assay for *N*-succinyl-DAP aminotransferase

E.C.1.4.1.4) as it is also specific for L-glutamate and α-ketoglutarate. The NADH-utilizing isozyme from animal sources (E.C.1.4.1.3) is less specific for the substrates it will accept and might show spurious activity especially with crude enzyme solutions. Its advantage is that it can utilise the much cheaper non-phosphorylated nicotinamide co-factor and might be perfectly acceptable for assaying purer enzyme extracts. Alternatively, an NADPH recycling system could be incorporated into the assay,[175] however, this additional complication was not introduced in this study.

A major concern in any coupled assay system is to ensure that the reaction rate of the coupling enzyme never becomes rate-limiting so that the observed activity is always a measure of the primary enzyme's rate. In assaying the aminotransferase activity, a large excess of the coupling enzyme was used (10 Units/mL). Increasing this concentration further did not change the rate of decrease of absorbance ($\Delta A_{340}$). The very large $K_{eq}$ for the conversion of α-ketoglutarate to glutamate ($2.2 \times 10^{13}$ $M^2$) ensures this reaction is essentially irreversible in spite of the much higher concentration of glutamate over α-ketoglutarate. $K_m$ values for the glutamate dehydrogenase are: $9.5 \times 10^{-4}$ M for α-ketoglutarate, $8.5 \times 10^{-5}$ M for NADPH, and 0.11 M for $NH_3$, in phosphate buffer, pH 8.0 at 30 °C.[176] Reported $K_m$ values for the aminotransferase in Tris-HCl buffer, pH 8.0 at 37 °C are: $5.0 \times 10^{-4}$ M for the α-keto substrate **50** and $5.2 \times 10^{-3}$ M for L-glutamate.[70]

Control experiments ensured that the aminotransferase substrates did not inhibit the coupling enzyme. Other controls were run to ensure that the observed activity was in fact due to the aminotransferase, by methodically deleting one component of the assay at a time and checking for activity. No activity was observed in the absence of either enzyme or substrate. Protein levels were determined by the Bradford method[177] using the Bio-Rad Protein Assay Reagent. Calibration curves were prepared with bovine serum albumin.

**Isolation of the Aminotransferase**

In the previous isolation of this enzyme[70,71] a crude extract from a lysine-requiring mutant of *E. coli* was obtained, with other aminotransferase activity still present. The isolated aminotransferase extract had a specific activity of 5.33 Units/mg (1 Unit = 1 μmol of substrate converted/min·mg). The isolation scheme consisted of a $MnCl_2$ precipitation step on the cell free extract to remove nucleic acids, followed by dialysis, a freeze and thaw cycle, and chromatography on DEAE-cellulose. The purification scheme used in our study is summarized in Figure **14**. In the absence of an overproducing organism we isolated the enzyme from *E. coli W* (wild type). Cells were grown under the same conditions as previously described[70] in a 10 L fermentor to afford 120 g of wet cells. A cell free extract in MOPS, pH 8 buffer was obtained by sonication and centrifugation of the cell debris. An accurate measure of the aminotransferase activity in this extract was not possible due to high background activity. The first few steps of the isolation reproduced the purification scheme used previously. Nucleic acid precipitation by addition of 1 M $MnCl_2$ to a final concentration of 50 mM was followed by dialysis with 50 mM Tris-HCl, pH8. The freeze-thaw step was omitted as the purification previously achieved in this step did not justify the loss of activity. The dialysate was applied to a DE-52 cellulose ion exchange column and eluted with a KCl gradient in 10 mM Tris-HCl, pH 8. A three-fold decrease in protein was observed, but unfortunately a similar loss of activity occurred, hence little gain was achieved in this step. A single broad band of protein came off the column (compared to the literature isolation where numerous protein peaks were eluted and an 11-fold purification was achieved). The recovered enzyme was frozen with added glycerol and PLP to increase stability. The next purification step was gel-filtration chromatography on Sephadex G-100 which has a fractionation range of 4,000-150,000 daltons. The sample from the DE-52 column was run down a 2.5 x 89 cm column in 7 batches. The protein elution profiles for the different batches were virtually identical. The protein emerged in one large drawn out

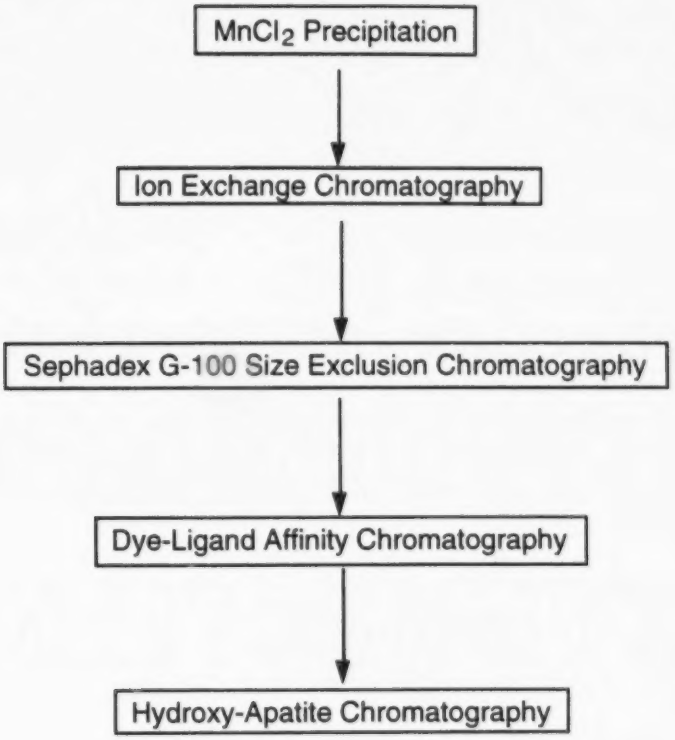



**Figure 14** Aminotransferase isolation scheme

band with the activity spread over 35 mL in the center of the band. Due to the diffuse nature of the active band, no attempt was made to estimate a molecular weight for the native enzyme from its retention time on the column. While the amount of protein in the active fractions was only 50% of that applied to the column, only 22% of the activity was recovered, representing a decrease in purification. At this point the enzyme had a lower specific activity than was present after the first step.

In order to rectify this situation a technique offering a more specific mode of action was sought. One such technique is dye-ligand affinity chromatography.[178,179] The most commonly used dyes are the Procion series (ICI) and the Cibacron dyes (Ciba Geigy), all of which have a polysulfonated chromophore linked to a triazine group which is bound to the solid support. The color is related to the type of structure, so blue dyes, for instance,

have an anthraquinone chromophore. These dyes are large highly functionalized flexible poly-cyclic compounds which can mimic the shape and polarity of numerous biomolecules and hence bind to proteins in place of these molecules. Cibacron Blue 3G-A (Figure **15**) has been shown by X-ray crystallography to behave as an analog of adenosine diphosphate-ribose[180] and will selectively bind to various nucleotide-binding proteins. Selective elution of specifically-bound proteins is then possible with the biomolecule being

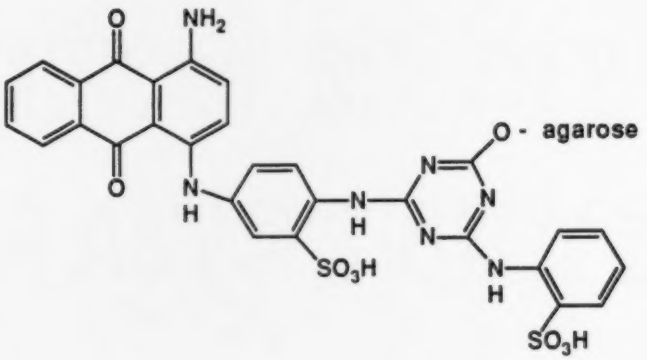



**Figure 15** Cibacron Blue 3G-A-Agarose Type 3000-CL

mimicked by the dye, in an example of affinity chromatography. As well as the site-specific interactions, hydrophobic, electrostatic, and hydrogen bonding interactions are also possible due to the variety of different structural features and functional groups found in these compounds. Stronger binding usually occurs at lower pH, due to the cation-binding effect of the negatively charged sulfonate groups, and at lower salt concentration.

Additional purification can be achieved by using a "negative"-binding dye column immediately in line before the "positive"-binding dye column. A negative dye is one which will not bind the protein of interest but will bind a significant amount of other protein present in the extract. This can greatly reduce the amount of protein binding to the second column and possibly remove other proteins that would have eluted with the desired protein from the positive column.

In order to reduce the number of dyes that need to be tested to find optimal binding of a protein, a classification system of the most commonly used dyes has been developed, based on the amount of protein bound from various crude biological extracts.[181] The dyes have been ordered into five groups according to their average protein-binding ability, with Group 1 binding the least protein and Group 5 binding the most. By selecting one dye from each group to test the "stickiness" of the enzyme in question it can be determined whether it binds to a "general" dye or to one which binds less overall protein. Once a binding dye is found, the other dyes in that group, and in the adjoining more-selective group, can be examined for optimal binding of the enzyme. Using this principle five agarose-bound dyes, all from different binding-groups, were tested for their ability to bind the aminotransferase in 50 mM Tris-HCl pH 7.5. The dyes tested were: Cibacron Blue 3G-A-Type 3000, Reactive Brown-10, Reactive Green-19, Reactive Yellow-86, and Reactive Blue-4. A summary of the results is shown in Figure **16**. The strongest activity-binding dyes were Blue-3 and Green-19, while Brown-10 appeared to have potential as a negative dye. Further studies on the Blue-3 and Green-19 dyes showed that better binding could be achieved at lower pH (6.0-6.5) in 30 mM phosphate. Washing the columns with either 30 mM DAP or 20 mM *N*-succinyl DAP did not elute the enzyme suggesting that non-specific binding is occurring. The enzyme could be eluted with a pH gradient, coming off at pH 7.0-7.5, or alternatively it could be eluted with 0.1-0.2 N NaCl. Higher recovery of the enzyme was obtained from the Blue-3 dye than from the Green-19 dye.

In the full-scale purification the enzyme from the Sephadex column was applied in 30 mM phosphate pH 6.5 to a column of Reactive Brown-10 connected in tandem with a column of Cibacron Blue-3G-A. After washing through with buffer the columns were separated and eluted. The protein eluted from the brown column was 25% of the total applied and contained only a small amount of activity. The enzyme was eluted from the

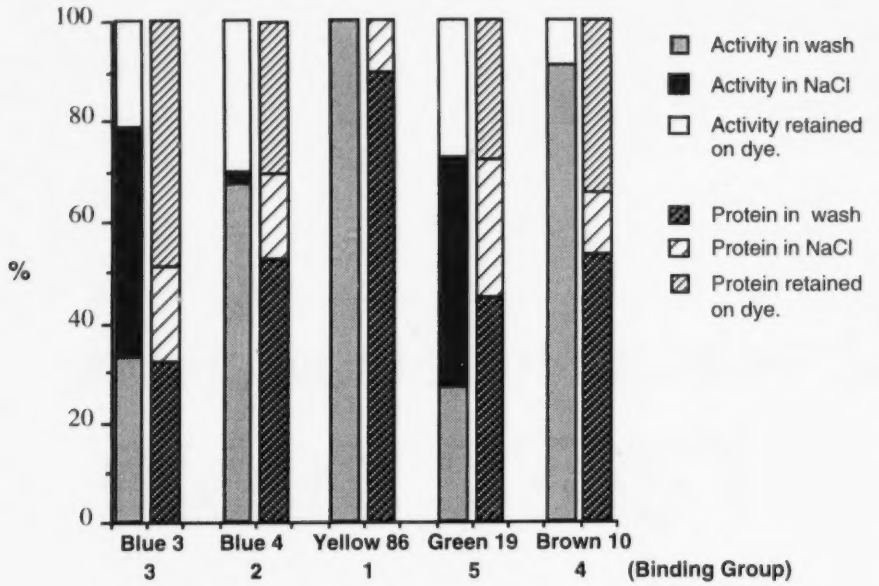
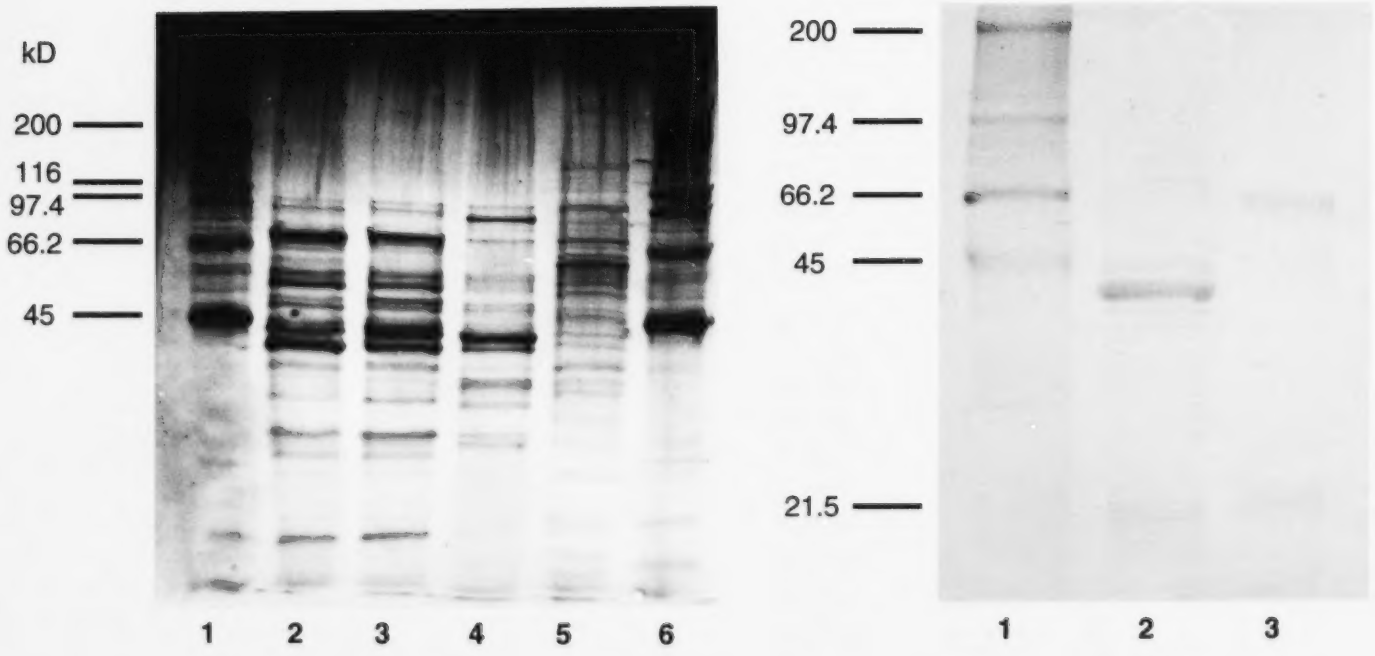



**Figure 16** Dye-ligand binding study

blue column with a pH gradient, coming off at pH 7.5-8.0. A subsequent wash with 0.2 N NaCl also eluted some activity. The total activity recovered from the column was 77% of that applied, with a 44-fold increase in purity. SDS-PAGE of the recovered enzyme showed a large decrease in the number of bands present with a major band occurring at 40 kdaltons (Figure **17a**).

The final purification step in this isolation was hydroxy apatite chromatography. Elution was achieved with a phosphate concentration gradient, the enzyme eluting at 0.2-0.3 M phosphate pH 6.8. This step achieved a doubling of the purity with the most active fraction being purified nearly four-fold. SDS-PAGE of this fraction (Figure **17b**) shows one dominant band with a few very faint additional bands. Estimation of the molecular weight of the dominant band by comparison to the molecular weight markers is 40,000 daltons. The purification results are summarized in Table **6**.




a) Lane 1. High M.W. markers
2. Dye Column - high activity
3. - lower activity
4. - NaCl eluate
5. Sephadex G - 100 activity
6. High M.W. markers

b) Lane 1. High M.W. markers
2. Hydroxy Apatite
3. Low M.W. markers

**Figure 17.** Silver stained SDS-PAGE gels of aminotransferase isolation

**Table 6** Aminotransferase purification summary

| Procedure | Volume mL | Activity Units | Protein mg | Specific Activity | Yield % | Fold Purif. |
|---|---|---|---|---|---|---|
| $MnCl_2$ | 120 | 1,570 | 2,300 | 0.683 | --- | --- |
| DE-52 | 13.3 | 442 | 570 | 0.775 | 28 | 1.13 |
| Seph. G-100 | 6.5 | 75.4 | 205 | 0.368 | 4.8 | 0.54 |
| Dye-Ligand | 4.9 | 58.3 | 3.58 | 16.3 | 3.7 | 23.9 |
| Hydroxy - Apatite | 2.4 | 43.8 | 1.48 | 29.6 | 2.8 | 43.3 |
| most active fraction | 0.35 | 8.47 | 0.14 | 60.5 | 0.54 | 88.6 |

In summary, the aminotransferase was substantially purified with the bulk of the purification occurring in two steps. Dye-ligand chromatography using a negative and a positive column in tandem was by far the most efficient purification step, achieving a 44-fold purification from the previous step. Chromatography on hydroxy-apatite also resulted in useful purification. The specific activity of the most active fraction was 60.5 Units/mg (µmol substrate converted/min·mg protein).

Based on the information obtained from this isolation, an improved purification procedure can be suggested. The steps prior to the dye ligand columns should be eliminated, at least at that stage of the purification. Instead, the purification should commence with the crude extract being applied immediately to the dye ligand columns in order to gain the maximum benefit from the high efficiency of this step. Subsequent chromatography on hydroxy apatite followed by Sephadex gel filtration should provide a

highly purified enzyme extract. The Sephadex step should also provide an estimate of the molecular weight of the native enzyme, thus determining its aggregation behaviour (eg dimer, tetramer etc). The continuing investigation of the enzyme within this group will focus on determination of the N-terminal amino acid sequence by transfer of the enzyme from an SDS-PAGE gel to a PVDF (polyvinylidenedifluoride) membrane followed by Edman degradation.[182,183] With this information a collaborative study is planned to locate and clone the gene that codes for the aminotransferase.

**Determination of Michaelis-Menten Constants**

It has previously been shown that enzyme steady-state kinetic mechanisms can be distinguished by visual inspection of initial rate plots.[184] It is characteristic of two-substrate enzymes having, in Cleland nomenclature,[185] a Ping Pong Bi Bi mechanism, that double reciprocal (Lineweaver-Burk) plots of velocity against one substrate concentration at fixed concentrations of the other substrate yield a series of parallel lines with slopes of $K_m^{app}$ / $V_{max}^{app}$. That this enzyme does indeed have a classical Ping Pong Bi Bi mechanism is demonstrated in Figure **18a** where the reciprocal plots at four fixed concentrations of glutamate are shown. Rearrangement of the data provides the plots for varied glutamate concentrations at fixed levels of the α-keto substrate **50** as shown in Figure **18b**. The original data was obtained at five concentrations of glutamate and seven concentrations of the α-keto substrate, however substrate inhibition was observed at the highest level of each substrate (50 mM and 5 mM respectively) so these values were not included in the analyses. Aminotransferases are known to be markedly susceptible to substrate inhibition by both the amino and oxoacid substrates.[186-190]

The apparent $K_m$ and $V_{max}$ values at each fixed substrate concentration were determined by computer using an iterative, robust , non-linear regression analysis (Enzyme Kinetics, Trinity Software, Compton NH). The relationship between the apparent and real $K_m$ and $V_{max}$ values is shown in the double reciprocal plots of the $K_m^{app}$ and $V_{max}^{app}$

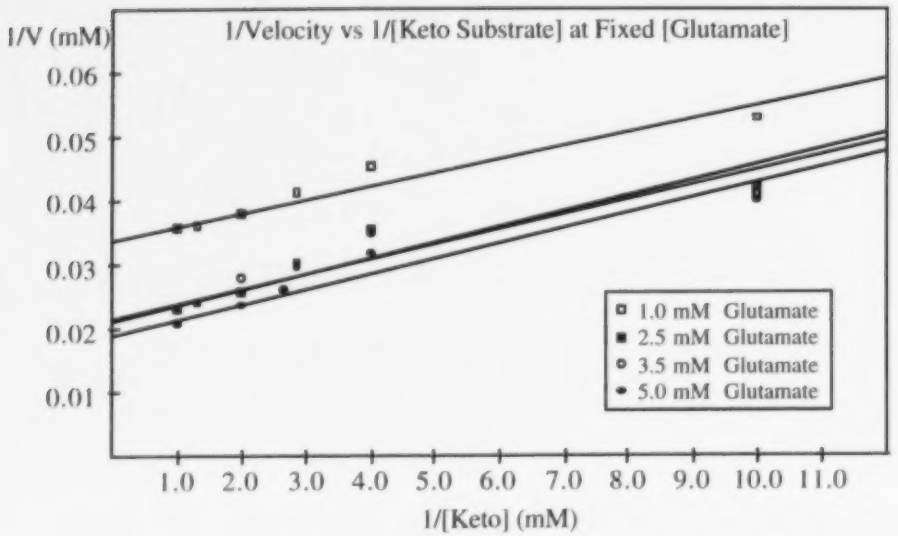



**Figure 18a** Lineweaver-Burk Plot (at fixed [glutamate])

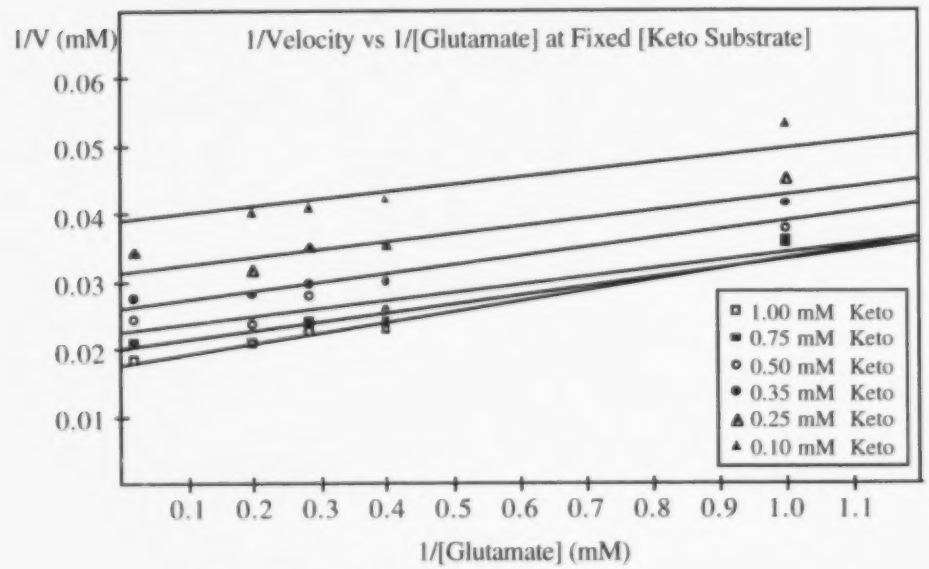



**Figure 18b**. Lineweaver -Burk Plot (at fixed [α-keto substrate])

values versus [glutamate] (Figures **19a** and **b** respectively). The replots against [α-keto substrate] (Figures **19c**, **d**) also allow calculation of values for the real $K_m$ and $V_{max}$ constants. The actual values for these constants, shown in Table **7**, were derived by non-linear regression analysis rather than the less statistically-valid double reciprocal plots.[191] The large errors associated with some of these values are due to a number of factors. The major reason is that the remaining substrate concentrations, after omission of the inhibiting highest concentrations, are too closely spaced to give a highly accurate curve fit. Reducing the number of data points from 35 to 24 also reduces the accuracy of each initial reciprocal plot. While the maximum substrate concentration is limited by the appearance of substrate inhibition the minimum concentration will be a function of the spectrophotometer's limit of measurement and the ability to detect an initial rate before the velocity starts decreasing.

The calculated Km values are lower than the literature values, which were determined from a plot of [S]/V versus [S] (Hanes-Woolf plot).[70] This may in part be due to the measurements being made on an enzyme preparation of different purity.

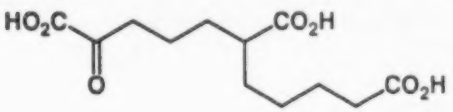
**Table 7** Michaelis-Menten constants

| Plot | $K_{m(glut)}$ (mM) | $K_{m(keto)}$ (mM) | $V_{max}$ (nmol/min·mg) |
|---|---|---|---|
| $K_m^{app}$ vs [glut] | 1.48 ± 0.89 | 0.18 ± 0.04 | ------- |
| $V_{max}^{app}$ vs [glut] | 1.12 ± 0.35 | -------- | 65.1 ± 5.8 |
| $K_m^{app}$ vs [keto] | 1.21 ± 0.14 | 0.26 ± 0.08 | ------- |
| $V_{max}^{app}$ vs [keto] | -------- | 0.20 ± 0.05 | 67.3 ± 5.2 |
| Literature values[70] (in Tris pH 8, 37°C) | 5.2 | 0.50 | ------- |

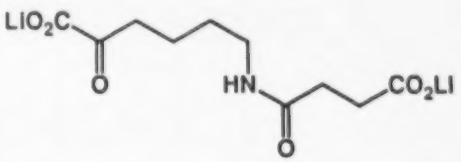
**Figure 19** $K_m$ and $V_{max}$ Replots

### α-Keto-Substrate Analog Studies

The desamino and descarboxy α-keto-substrate analogs (**5** and **6** respectively) were assayed with the aminotransferase, initially in the absence of the α-keto substrate **50** in order to observe any turnover, and then with the substrate present to determine if they inhibited substrate turnover. The results are shown in Table **8**. The descarboxy analog **6**




**5**




**6**

was not turned over by the enzyme whereas the desamino analog **5** had a low turnover rate. Neither compound showed any inhibition of substrate turnover at the concentration tested. These results suggest that the 7-carboxylate group is essential for binding in the active site while the carboxamide group is less important. In work being continued by Dr. Russell Cox, the *N*-acetyl-α-keto substrate analog is being synthesized for testing. If this compound has a high rate of conversion by the enzyme it may prove to be a more convenient substrate for the assay than the natural substrate, since it lacks the unstable succinamide group.

**Table 8** Effects of α-keto substrate analogs on enzyme activity

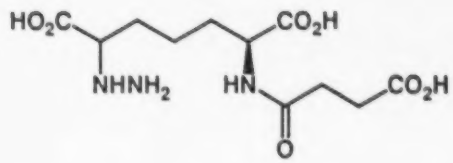
| **Substrate Analog** | **Concentration** mM | **Activity** % | **Substrate (50)** mM | **Inhibition** % |
|---|---|---|---|---|
| Desamino (**5**) | 7.5 | 19 | 0.58 | 0 |
| Descarboxy (**6**) | 4.9 | 0 | 0.51 | 0 |
| 2-HTHP | 1.0 | 41 | 1.00 | -- |

The tetrahydrodipicolinate analog, 2-hydroxytetrahydropyran-2,6-dicarboxylate (2-HTHP) is a potent competitive inhibitor of tetrahydrodipicolinate *N*-succinylase, the enzyme responsible for the step immediately prior to the aminotransferase step in the DAP pathway.[68] In aqueous solution this compound is an approximately equal mixture of the open-chain and cyclized tautomers (Figure **20**). In the open-chain form this compound represents an α-keto substrate analog for the aminotransferase, with the succinamide being replaced by a hydroxyl group. A small amount of the racemic material was obtained[192] for testing in the aminotransferase assay and showed a significant turnover rate (Table **8**), thereby demonstrating that the succinamide group can be replaced by another polar group and still be a substrate for the enzyme. There was insufficient material to obtain a $K_m$ value for this compound. As the 6*R*-enantiomer has the opposite stereochemistry compared to the natural substrate, presumably only the 6*S*-enantiomer is a substrate for the enzyme, and should show a rate double that of the racemic mixture when tested separately, making it comparable to the natural substrate's rate. The 6*S*-enantiomer is currently being synthesized by Dr. Russell Cox.

**Figure 20** 2-Hydroxytetrahydropyran-2,6-dicarboxylate (2-HTHP)

**Inhibitor Studies**

The *N*-amino product analog **3** was tested for inhibition of the aminotransferase at three concentrations from 1 mM to 10 μM (Table **9**) at an α-keto substrate concentration of 1 mM. Hydrazine and hydroxylamine were also tested for comparison. As all these



3

**Table 9** Inhibition Results

| Compound | % Inhibition* at: | | |
|---|---|---|---|
| | 1 mM | 100 μM | 10 μM |
| $NH_2NH_2$ | 98 | 29 | 0 |
| *N*-amino analog **3** | >99 | 65 | 16 |
| $NH_2OH$ | >99 | 71 | 15 |

* At [50] = 1 mM.

compounds act through formation of stable complexes with PLP, it was necessary to reduce the exogeneous PLP level in the assay mixture. At 1 mM all the compounds showed virtually complete inhibition of the enzyme. The *N*-amino analog **3** was an even stronger inhibitor than hydrazine, presumably due to stronger binding in the active site. It still caused some inhibition at 100-fold dilution when hydrazine had lost all effect. Hydroxylamine had a very similar inhibition profile to the *N*-amino analog.

In conclusion, an approach to stereoselective α-alkylation of α-amino acid esters has been investigated; an existing synthesis of the DAP stereoisomers and their selectively protected forms has been improved; and a new method for synthesis of the LL- and D,D-DAP isomers in high diastereomeric excess has been developed. *N*-Succinyl diaminopimelate aminotransferase has been purified from *E. coli*. Its kinetic constants have been determined and a possible subunit molecular weight has been estimated. Several enzyme substrate- and product-analogs were synthesized and tested for inhibition of the enzyme; of these, the *N*-amino-product analog showed strong inhibition.

## EXPERIMENTAL

### General

Reactions requiring anhydrous conditions or involving air-sensitive reagents were performed under a positive pressure of argon (Ar) using oven-dried (>6 h, 130 °C) glassware which had been cooled under Ar. The removal of solvent *in vacuo* refers to evaporation on a rotary evaporator followed by drying under high vacuum (<1 mm Hg) to constant sample weight. Solvents for anhydrous reactions were dried according to Perrin *et al.*[193] Benzene, THF, diethyl ether, and toluene were distilled from sodium and benzophenone. Triethylamine, diisopropylamine, pyridine, HMDS, DMPU,[143] chloroform, dichloromethane, and carbon tetrachloride were distilled from calcium hydride. Anhydrous methanol and ethanol were distilled from magnesium metal and catalytic iodine. Ethanol-free chloroform was obtained by percolation through a column of activated basic alumina (Grade 1, 10 g/14 mL). Solvents used for chromatography were distilled. Water used was Milli-Q (Millipore) quality.

All reagents used were of ACS grade or finer. (2*R*)-(+)- and (2*S*)-(-)-1-benzoyl-2-*tert*-butyl-3-methyl-4-imidazolidinone were obtained from Fluka Chemie (Switzerland). (3*S*)-(+)-3,6-dihydro-2,5-dimethoxy-3-isopropylpyrazine was supplied by Merck Schuchardt (Germany) and was distilled before use. Amino acids and their derivatives were purchased from Sigma Chemical Co. Diazomethane ($CH_2N_2$) was prepared using Diazald™. All commercial organometallic reagents were obtained from Aldrich Chemical Co. BuLi was periodically titrated against menthol / phenanthroline.[194] LiHMDS and KHMDS were titrated against menthol / fluorene before use. KHMDS was prepared according to the procedure of Brown.[195] Marfey's reagent (*N*-1-(2,4-dinitro-5-fluorophenyl)-L-alanine amide) was prepared according to the literature procedure.[120]

Whenever possible, the progress of reactions was monitored by thin-layer chromatography (TLC) using one or more of the following methods for visualization: UV

absorption by fluorescence quenching, iodine staining; dodecaphosphomolybdic acid / $Ce(SO_4)_2$ spray for general hydrocarbons; ninhydrin spray for amino acids and amines; bromocresol green spray for acids; and 2,4-dinitrophenylhydrazine or *o*-phenylenediamine sprays for α-keto acids. All spray reagents were prepared and used as described by Krebs *et al.*[196] When necessary, plates were developed by heating with a hot-air gun. TLC of amino acids on cation exchange resin used sodium citrate buffer (pH 5.0) or 0.5 N $NH_4Cl$ as solvents. TLC of amino acids on reverse phase silica used either n-BuOH:AcOH:$H_2O$ (2:1:1) or $^i$PrOH:conc. aqueous $NH_3$:$H_2O$ (7:2:1) as solvents. Commercial thin layer and preparative thin layer chromatography plates used were: normal silica, Merck 60 $F_{254}$; reverse-phase, Merck RP-8 and 18 $F_{254}S$; cation exchange resin, Polygram® Ionex-25 SA-Na. Flash chromatography was performed according to the method of Still[197] with the modification that stepwise solvent-polarity gradients were often used, with Merck type 60, 230-420 mesh silica gel and argon pressure. All solvent mixtures listed were volume ratios. BioRad cation and anion exchange resins were used for ion exchange chromatography. Microcrystalline cellulose for column chromatography was purchased from Merck.

All literature compounds had $^1$H NMR, IR, and mass spectra (MS) consistent with the assigned structures. Melting points are uncorrected and were determined on a Büchi oil immersion apparatus using open capillary tubes. IR spectra were recorded on Nicolet 7199 or 20SX FT-IR spectrometers. Cast refers to the evaporation of a solution on to a NaCl plate. Mass spectra were recorded on a Kratos AEI MS-50 (high resolution, electron impact ionization), MS-12 (chemical ionization; CI, $NH_3$), and MS-9 (fast atom bombardment with argon, posFAB) instruments. Cleland matrix used in posFAB spectra refers to a 5:1 mixture of dithiothreitol and dithioerythritol. Microanalyses were obtained on Perkin Elmer 240 or Carlo Erba 1108 elemental analyzers. Optical rotations were measured on a Perkin Elmer 241 polarimeter using a microcell (10.0 cm path length, 0.9 mL volume) at ambient temperature. All specific rotations reported were measured at the

sodium D line and are referenced against air. Nuclear magnetic resonance (NMR) spectra were measured on Bruker WP-80 (continuous wave), WH-200, AM-300, WM-360, or WH-400 instruments. Tetramethylsilane (TMS) was used as an internal standard for $^{1}H$ NMR spectra. $^{13}C$ NMR spectra were referenced to the deuterated solvent peak, or for samples in $D_2O$, acetonitrile or 1,4-dioxan was added as an internal reference..

High pressure liquid chromatography (HPLC) was performed on a Beckman System Gold chromatograph equipped with a 126 solvent module, 166 variable wavelength detector, and an Altex 210A injector with a 100 µL sample loop; with a ChemcoPak® Chemcosorb 5-ODS-UH (4.6 x 150 mm) column obtained from Dychrom (Sunnyvale CA). Solvents were degassed by passage through a 0.45 µm filter under vacuum.

The biochemicals for the enzyme work were obtained from Sigma. Dialysis was performed with Spectropor tubing sizes 2 and 4 obtained from Fisher. DEAE cellulose was obtained from Whatman while the immobilized dyes and the Sephadex gel were purchased from Sigma. Hydroxy apatite for chromatography was BioRad Bio-Gel HTP. Ultrafiltration was performed with an Amicon Stirred Ultrafiltration Cell, Model 8200, with a YM-10 membrane (10,000 MW cut-off), under nitrogen pressure. Column chromatography was performed with BioRad Econo-Columns and fittings. Centrifugation was performed at 4 °C with a Sorvall® RC-5B centrifuge equipped with SS-34, SM-24, GSA, and GS-3 rotors. Spectrophotometric assays were performed on a Hewlett Packard (HP) 8452A diode array spectrophotometer controlled by a HP Vectra ES computer loaded with HP89530A MS DOS-UV/VIS operating software, and equipped with a water-jacketed single cell holder. Electrophoresis was performed with a BioRad MinProtean vertical slab unit and a BioRad 3000 Xi computer-controlled power supply, using precast BioRad Ready Gels (1mm thickness, 12%, single percentage, 0.375 M Tris-HCl). The gels were stained with BioRad Silver Stain according to its accompanying procedure. Proteins run as molecular weight markers were obtained from Sigma as separate mixtures of high and low molecular weight standards.

Compounds labelled with a [§] superscript were prepared by Dr. Russell J. Cox of this research group; compounds labelled with a [¶] superscript were prepared by Dr. Lister K.P. Lam, a former post-doctoral fellow in this research group.

**(6*S*)-2*N*-Amino-6-*N*-succinyldiaminopimelic Acid (3)**

The general procedure of Benigni and Dickson[163] was adapted. Hydrazine monohydrate (98%, 90.0 μL, 1.80 mmol) was added to a stirred solution of **50** (64.1 mg, 0.208 mmol) in MeOH (5 mL) and the solution was stirred at room temperature for 3 h. It was then diluted with water (2 mL) and passed through a short column of C-18 reverse phase silica (Waters Sep-pak, 1.0 g) to remove the remaining hydrazine. The MeOH was removed *in vacuo*, 3% Na/Hg amalgam (1.0 g) was added to the aqueous solution, and the mixture was stirred for 12 h under argon. The aqueous layer was removed by pipette and acidified to pH 3 with 1 N HCl. Concentration *in vacuo* gave a white solid which was dissolved in water (2 mL), neutralised with saturated $NaHCO_3$ and applied to a cation exchange column (Biorad AG50W-X8, $H^+$ form, 1.5 x 15 cm) which was washed with water (100 mL) and eluted with 2 N $NH_4OH$ (100 mL). Concentration *in vacuo* and lyophilization of the ninhydrin positive fractions gave **3** (15.2 mg, 24%) as a white solid: $^1$H NMR (400 MHz, $D_2O$) δ 4.10–4.05 (m, 1H, C**H**NHC(O)), 3.40-3.33 (m, 1H, C**H**$N_2H_4$), 2.50-2.37 (m, 4H, succinyl C**H**$_2$C**H**$_2$), 1.80-1.57 (b m, 4H, C**H**$_2$C**H**$_2$C**H**$_2$), 1.40-1.30 (m, 2H, $CH_2$C**H**$_2$$CH_2$); $^{13}$C NMR (100 MHz, $D_2O$ + $CH_3CN$) δ 181.7 (**C**$O_2$H), 179.8 (**C**$O_2$H), 175.7 (**C**$O_2$H), 66.5 (**C**H$N_2H_4$), 55.4 (**C**HNHC(O)), 33.5 (C**H**$_2$), 32.0 (C**H**$_2$), 29.9 (C**H**$_2$), 26.1 (C**H**$_2$), 22.1 (C**H**$_2$); MS (posFAB, Cleland) 305.96 (306.13 calcd for $C_{11}H_{20}N_3O_7$, $MH^+$).

**6-Carboethoxy-2-oxoundecan-1,11-dioic Acid** [§¶] **(5)**

A suspension of **54a/b** (mixture of diesters, 49.0 mg, 0.146 mmol based on an averaged molecular weight of 336) in 1 N HCl (5 mL) was heated to 100 °C for 2 h with stirring, during which time it gradually became homogeneous. The mixture was cooled to room temperature, the solvent was removed *in vacuo*, and the residue was redissolved in water and lyophilized to give **5** (39.9 mg, quantitative) as a colorless oil: $^1$H NMR (300 MHz, acetone-$d_6$) δ 9.55 (b s, 3H, $CO_2$**H**), 2.92-2.86, (m, 2H, C**H**$_2$C(O)$CO_2$H), 2.40-2.33 (m, 1H, C**H**$CO_2$H), 2.28 (t, 2H, *J* = 7.4 Hz, C**H**$_2CO_2$H), 1.68-1.27 (b m, 10H, 5 x C**H**$_2$); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 196.0, 177.3, 175.0, 162.4, 45.6, 38.8, 34.0, 32.6, 32.1, 27.6, 25.5, 21.5; MS (posFAB, Cleland) 275 ($MH^+$).

**6-*N*-Succinylamino-2-oxohexanoic Acid, Tri-Lithium Salt (6)**

The dimethyl ester **58** (7.61 mg, 27.8 μmol) was dissolved in a solution of lithium hydroxide monohydrate (2.33 mg, 55.6 μmol) in acetonitrile/water (50:50, 0.5 mL) and stirred vigorously for 2 h at room temperature. The acetonitrile was removed *in vacuo* and the aqueous residue was lyophilized to give **6** (5.80 mg, 81%) as a pale yellow amorphous solid: IR (KBr disk) 3430 (b), 2931, 1636 (b), 1417 $cm^{-1}$; $^1$H NMR (400 MHz, $D_2O$) δ 3.13 (t, 2H, *J* = 6.7 Hz, C**H**$_2$NH), 2.71 (t, 2H, *J* = 7.1 Hz, C**H**$_2$C(O)$CO_2$), 2.39 (s, 4H, succinyl C**H**$_2$C**H**$_2$), 1.58-1.42 (bm, 4H, 2 x C**H**$_2$); $^{13}$C NMR (100 MHz, $D_2O$ + MeOH) δ 39.1, 38.8 (2 x succinyl C**H**$_2$), 33.3, 32.6, 27.9, 20.14 (4 x C**H**$_2$); MS (posFAB, Cleland) 275 ($MNH_4^+$), 258 ($MH^+$).

**3α, 12α, 23-Trihydroxy-5β-cholane (7)**

A solution of deoxycholic acid (20.0 g, 50.9 mmol) in dry THF (200 mL) was added dropwise to a suspension of lithium aluminum hydride (5.81 g, 153 mmol) in THF (500 mL) under argon. The mixture was heated to reflux for 2.5 h and then cooled. Ice/water (100 mL) was added slowly with stirring followed by cold 10% $H_2SO_4$ (150 mL)

The aqueous layer was extracted with ether (4 x 200 mL). The combined organic layers were washed with 5% $Na_2CO_3$ (300 mL), dried over $MgSO_4$, and concentrated *in vacuo* until crystallisation commenced. Cooling and filtration provided **7** (9.02 g, 47%) as white crystals: mp 117-130 °C (lit.[113] mp 106-118, 123 °C; lit.[114] mp 90-120 °C); IR (MeOH cast) 3350, 2956, 2890, 1449, 1377, 1043 $cm^{-1}$; $^1H$ NMR (200 MHz, $CD_3OD$) δ 3.96 (t, 1H, $J$ = 0.3 Hz, C-12: **H**β), 3.62–3.42 (m, 1H, C-3: **H**β), 3.50 (t, 2H, $J$ = 6.9 Hz, C-24: C**H**$_2$), 2.00-1.69 (m, 7H, C-1,4,9,16,17:**H**β; C-6: **H**β; C-22: **H**$_a$H), 1.68-1.26 (m, 13H, C-6,14: **H**α; C-2,4,5,7,8,15,16: **H**β; C-20: C**H**; C-22: CH**H**$_b$; C-11: C**H**$_2$), 1.26-0.90 (m, 2H, C-7,15: **H**α), 1.01 (d, 3H, $J$ = 6.6 Hz, C-21: C**H**$_3$), 0.92 (s, 3H, C-19: C**H**$_3$), 0.70 (s, 3H, C-18: C**H**$_3$); exact mass 360.3035 (360.3028 calcd for $C_{24}H_{40}O_2$, $M^+$-$H_2O$); MS (CI, $NH_3$) 396 ($MNH_4^+$), 379 ($MH^+$).

**3α, 12α-Dihydroxy-24-*p*-toluenesulfonyloxy-5β-cholane (8)**

To a suspension of **7** (7.57g, 20.0 mmol) in dry $CH_2Cl_2$ (600 mL) under argon was added *N,N*-4-dimethylaminopyridine (2-3 crystals) and $Et_3N$ (13.9 mL, 100. mmol) with cooling in an ice bath. A solution of *p*-toluenesulfonyl chloride (*p*-tosyl chloride, 3.81 g, 20.0 mmol) in $CH_2Cl_2$ (10 mL) was then added dropwise and the mixture was slowly warmed to room temperature and stirred for 24 h. More *p*-tosyl chloride (1.91 g, 10.0 mmol) was added and stirring continued for a further 64 h. The mixture was poured into 1% $NaHCO_3$ (250 mL) and the aqueous layer extracted with $CHCl_3$ (2 x 100 mL). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo* to give a yellow oil. Purification by flash chromatography (5 x 25 cm, 0-5% MeOH/$CH_2Cl_2$) afforded **8** (6.84 g, 64%) as a white foam: IR ($CH_2Cl_2$ cast) 3390, 2935, 2884, 1361, 1176 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 7.79 (d, 2H, J = 8.3 Hz, *o*-Ar**H**), 7.34 (d, 2H, $J$ = 8.3 Hz, *m*-Ar**H**), 4.03-3.93 (m, 1H, C-12: **H**β), 4.00 (t, 2H, $J$ = 6.4Hz, C-24: C**H**$_2$), 3.70-3.56 (m, 1H, C-3: **H**β), 2.46 (s, 3H, ArC**H**$_3$), 1.90-0.94 (b m, 26 H, 6 x C**H**, 10 x C**H**$_2$), 0.91 (d, 3H, $J$ = 6.3 Hz, C-21: C**H**$_3$), 0.90 (s, 3H, C-19: C**H**$_3$), 0.64 (s, 3H,

C-18: $CH_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 144.6, 133.3, 129.8, 127.9, 73.1, 71.8, 71.1, 48.2, 47.4, 46.4, 42.0, 36.4, 36.0, 35.2, 35.0, 34.1, 33.6, 31.3, 30.4, 28.6, 27.4, 27.1, 26.1, 25.6, 23.6, 23.1, 21.6, 17.4, 12.7; exact mass 514.3125 (514.3117 calcd for $C_{31}H_{46}SO_4$, $M^+$-$H_2O$); MS (CI, $NH_3$) 550 ($MNH_4^+$).

**3α, 12α-Dihydroxy-5β-cholane (9)**

To a stirred suspension of lithium aluminum hydride (2.16 g, 57.0 mmol) in dry THF (170 mL) under argon was added dropwise a solution of the tosylate **8** (7.59 g, 14.2 mmol) in THF (30 mL). The mixture was heated to reflux for 2 h and then stirred at room temperature for 12 h. More lithium aluminum hydride (1.08 g, 28.5 mmol) was added, and the mixture was heated to reflux for a further 12 h. Water (30 mL) was added slowly, followed by cold 1 N $H_2SO_4$ (100 mL) until all the solid had dissolved. The biphasic mixture was separated and the aqueous layer was extracted with $CHCl_3$ (2 x 150 mL). The combined organic layers were washed with 5% $Na_2CO_3$ (100 mL), dried over $MgSO_4$, and concentrated *in vacuo* to a white foam (5.18 g). Purification by flash chromatography (5 x 25 cm, 0-5% MeOH/$CH_2Cl_2$) followed by recrystallisation from $CH_3CN$ gave **9** (4.01 g, 77%) as large white crystals: mp 167.5-169.0 °C (lit.[112] mp 174-175 °C); IR ($CHCl_3$ cast) 3370, 2934, 2955, 1040 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$) δ 4.03-3.96 (m, 1H, C-12: $H_\beta$), 3.68–3.56 (m, 1H, C-3: $H_\beta$), 1.96-0.85 (b m, 26H, 6x**CH**, 10x$CH_2$), 0.95 (d, 3H, $J$ = 6.4 Hz, C-21: $CH_3$), 0.91 (s, 3H, C-19: $CH_3$), 0.88 (t, 3H, $J$ = 6.9 Hz, C-24: $CH_3$), 0.68 (s, 3H, C-18: $CH_3$); $^{13}C$ NMR (90 MHz, $CD_3OD$) δ 74.3, 72.8, 49.5, 48.7, 47.8, 43.9, 39.8, 37.7, 37.5, 37.2, 36.7, 35.6, 35.1, 31.4, 30.2, 29.0, 28.7, 27.7, 25.1, 24.0, 20.6, 18.3, 15.1, 13.5; exact mass 344.3081 (344.3079 calcd for $C_{24}H_{40}O$, $M^+$-$H_2O$), MS (CI, $NH_3$) 380 ($MNH_4^+$). Anal. Calcd for $C_{24}H_{42}O_2$: C, 79.50; H, 11.67. Found: C, 79.31; H, 11.40.

**Deoxycholic Acid Diacetate (10)**

The procedure of Ahmed *et al* [112] was modified. A mixture of deoxycholic acid (19.6 g, 50.0 mmol), dry pyridine (40.0 mL, 0.494 mol), and acetic anhydride (80.0 mL, 0.848 mol) was heated to reflux with stirring for 19 h with exclusion of moisture. The mixture was cooled, poured into ice/water (330 g), and extracted with ether (4 x 120 mL). The combined organic layers were washed with 2 N HCl (3 x 80 mL) followed by repeated washes with 5% $NaHCO_3$ until neutral pH was reached. The organic layer was dried over $Na_2SO_4$ and concentrated *in vacuo* to a yellow foam (25.2 g, theor. yield = 23.8 g). A sample was purified for characterization by flash chromatography (0-4% MeOH/$CHCl_3$) giving **10** as a white foam : IR ($CHCl_3$ cast) 3400, 1734, 1706 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 5.09 (b s, 1H, C-12: **H**β), 4.70 (m, 1H, C-3: **H**β), 2.50 -2.15 (m, 2H, C-23: C**H**$_2$), 2.11 (s, 3H, C**H**$_3CO_2$), 2.04 (s, 3H, C**H**$_3CO_2$), 1.95-0.98 (b m, 24H, 6xC**H**, 9xC**H**$_2$), 0.91 (s, 3H, C-19: C**H**$_3$), 0.82 (d, 3H, $J$ = 6.0 Hz, C-21: C**H**$_3$), 0.73 (s, 3H, C-18: C**H**$_3$); $^{13}C$ NMR (75 MHz, $CDCl_3$) δ 179.8, 170.6, 170.4, 75.9, 74.2, 49.4, 47.6, 45.1, 41.8, 35.7, 34.7, 34.6, 34.4, 34.0, 32.3, 30.9, 30.6, 27.3, 26.9, 26.6, 25.9, 25.6, 23.4, 23.1, 21.4, 21.3, 17.5, 12.4; exact mass 416.2931 (416.2926 calcd for $C_{26}H_{40}O_4$, $M^+$-$CH_3CO_2H$), MS (posFAB, Cleland) 477.04 (477.32 calcd for $C_{28}H_{45}O_6$, $MH^+$).

**3α, 12α-Diacetoxy-23-iodo-24-nor-5β-cholane (11)**

The literature preparation was followed.[112] A solution of **10** (24.72 g, 98% of the crude material) and $Pb(OAc)_4$ (95%, 25.7 g, 55.1 mmol) in dry $CCl_4$ (175 mL) under argon was heated to reflux for 15 min with irradiation by a 500 W tungsten lamp. Iodine (13.0 g, 51.2 mmol) in $CCl_4$ (600 mL) was then added dropwise over 2 h and stirring at reflux with irradiation was continued for a further 3 h. The solution was filtered, washed with 5% sodium thiosulfate (2 x 100 mL) to remove iodine and 5% NaOH (2 x 100 mL) to remove any unreacted acid. The organic layer was dried over $Na_2SO_4$ and concentrated *in*

*vacuo* to give a white foam which was purified by flash chromatography (10 x 15 cm, 15% EtOAc/hexanes) to give **11** (19.7 g, 72% from deoxycholic acid) as a white foam: IR ($CHCl_3$ cast) 2939, 2869, 1735, 1244, 1028 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 5.09 (t, 1H, *J* = 2.5 Hz,C-12: $H_\beta$), 4.79–4.64 (m, 1H, C-3: $H_\beta$), 3.35-3.24 (m, 1H, C-23: $CH_aHI$), 3.09-3.00 (m, 1H, C-23: $CHH_bI$), 2.10 (s, 3H, $CH_3CO_2$), 2.03 (s, 3H, $CH_3CO_2$), 2.00-0.98 (b m, 24H, 6x**CH**, 9x$CH_2$), 0.91 (s, 3H, C-19: $CH_3$), 0.82 (d, 3H, *J* = 6.0 Hz, C-21: $CH_3$), 0.75 (s, 3H, C-18: $CH_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 170.4, 170.3, 75.7, 74.1, 49.3, 47.4, 45.0, 41.7, 40.0, 36.4, 35.6, 34.6, 34.3, 33.9, 32.2, 27.3, 26.8, 26.5, 25.8, 25.5, 23.3, 23.0, 21.4, 21.3, 17.0, 12.3, 4.7; exact mass 498.1995 (498.1995 calcd for $C_{25}H_{39}IO_2$, $M^+$-$CH_3CO_2H$), MS (CI, $NH_3$) 576 (100%, $MNH_4^+$). Anal. Calcd for $C_{27}H_{43}IO_4$: C, 58.06; H, 7.76. Found: C, 58.44; H, 7.83.

**3α, 12α-Dihydroxy-24-nor-5β-cholane (12)**

Lithium triethylborohydride (220 mL of a 1 M solution in THF, 220 mmol) was added via syringe over 0.5 h to an ice cooled solution of **11** (18.9 g, 33.8 mmol) in dry THF (60 mL) under argon. The solution was stirred at room temperature for 4 h then treated with water (50 mL) and acidified to pH 1-2 with 1N HCl. The organic layer was separated and the aqueous layer was extracted with $CHCl_3$ (3 x 50 mL). The combined organic layers were washed with 5% sodium thiosulfate (200 mL) to remove iodine, washed with water (100 mL), and dried over $Na_2SO_4$. Concentration *in vacuo* gave a white foam which was purified by flash chromatography (10 x 15 cm, 20-50% EtOAc/hexanes) to afford **12** (11.6 g, 98%) as a white foam: IR (MeOH cast) 3350, 2940, 2870, 1448, 1377, 1036 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$) δ 3.96 (t, 1H, *J* = 2 Hz, C-12: $H_\beta$), 3.58-3.47 (m, 1H, C-3: $H_\beta$), 1.84-0.94 (b m, 26H, 6x**CH**, 10x$CH_2$), 0.91 (d, 3H, *J* = 6.4 Hz, C-21: $CH_3$), 0.87 (s, 3H, C-19: $CH_3$), 0.81 (t, 3H, *J* = 7.4 Hz, C-23: $CH_3$), 0.64 (s, 3H, C-18: $CH_3$); $^{13}C$ NMR (90 MHz, $CDCl_3$) δ 73.2, 71.7, 48.2, 47.3, 46.4, 42.1, 36.6, 36.4, 36.0, 35.3, 34.1, 33.6, 30.5, 28.5, 28.2, 27.5, 27.1, 26.1, 23.7, 23.1, 17.0,

12.7, 10.5; exact mass 348.3012 (348.3028 calcd for $C_{23}H_{40}O_2$). Anal. Calcd for $C_{23}H_{40}O_2$: C, 79.25; H, 11.57. Found: C, 79.18; H, 11.58.

**_N_-Benzoylglycine, 3α-(12α-Hydroxy)-24-nor-5β-cholanyl Ester (13)**

A solution of *N*-benzoylglycine (21.9 mg, 0.120 mmol) and **12** (34.8 mg, 0.100 mmol) in dry $CH_2Cl_2$ (1 mL), under argon, was treated with triethylamine (34.8 μL, 0.250 mmol). Bis-*N*,*N*-(2-oxo-3-oxazolidinyl)-phosphinic chloride (BOP-Cl, 31.5 mg, 0.120 mmol) was added as a solid and stirring was continued under argon at room temperature for 34 h. Aqueous 5% $NaHCO_3$ (1 mL) was added and the aqueous layer was then extracted with $CHCl_3$ (2 x 3 mL). The combined organic layers were dried over $Na_2SO_4$ and concentrated *in vacuo* to give a yellow oil. Purification by preparative TLC (silica gel, 50% EtOAc/hexanes) gave **13** (38.0 mg, 74%) as a clear oil: IR ($CHCl_3$ cast) 3340, 2937, 1735, 1653, 1540, 1210 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 7.85-7.78 (m, 2H, *o*-Ar**H**), 7.57-7.38 (m, 3H, *m,p*-Ar**H**), 6.80-6.70 (m, 1H, N**H**), 4.93-4.74 (m, 1H, C-3: $H_\beta$), 4.20 (d, 2H, *J* = 5.0 Hz, NHC$\mathbf{H}_2$CO$_2$), 4.03-3.98 (m, 1H, C-12: $\mathbf{H}_\beta$) 2.01-0.98 (b m, 24H, 6xC**H**, 9xC$\mathbf{H}_2$), 0.96 (d, 3H, *J* = 6.4 Hz, C-21: C$\mathbf{H}_3$), 0.93 (s, 3H, C-19: C$\mathbf{H}_3$), 0.84 (t, 3H, *J* = 7.2 Hz, C-23: C$\mathbf{H}_3$), 0.69 (s, 3H, C-18: C$\mathbf{H}_3$); $^{13}C$ NMR (90MHz, $CDCl_3$) δ 169.6, 167.3, 133.8, 128.5, 127.1, 76.0, 73.2, 48.2, 47.3, 46.4, 42.1, 41.9, 36.6, 36.0, 34.8, 34.1, 33.6, 32.1, 28.5, 28.1, 27.4, 26.9, 26.4, 26.0, 23.6, 23.0, 17.1, 12.7, 10.3; exact mass 509.3510 (509.3505 calcd for $C_{32}H_{47}NO_4$).

**_N_-Benzoyl-L-alanine, 3α-(12α-Hydroxy)-24-nor-5β-cholanyl Ester (14)**

A stirred suspension of *N*-benzoyl-L-alanine (predried over $P_2O_5$, 386 mg, 2.00 mmol) in dry $CH_3CN$ (5 mL) under argon in a glove box was treated with 1,1'-carbonyldiimidazole (0.400 g, 2.47 mmol). The solid dissolved with effervescence. After 5 min a white solid precipitated. Distilled allyl bromide (0.860 mL, 10.0 mmol) was added and stirring was continued for 0.5 h, at which point a solution of the sterol **12** (0.697 g, 2.00 mmol) in dry

$CH_2Cl_2$ (5 mL) was added. The white suspension was stirred for 60 h, complete dissolution occurring within 12 h. The solvent was removed *in vacuo* giving a white foam which was purified twice by flash chromatography (5 x 15 cm), initially with 3% $CH_3CN/CHCl_3$, and subsequently with 10-25% EtOAc/hexanes to afford **14** (0.436 g, 42%, white foam) as a diastereomeric mixture: IR ($CHCl_3$ cast) 3350, 2937, 1727, 1650, 1646, 1211 $cm^{-1}$; $^1H$ NMR (360 MHz, $C_6D_6$) δ 7.85-7.81 (m, 2H, *o*-Ar**H**), 7.14-7.03 (m, 3H, *m,p*-Ar**H**), 6.80 (d, 0.5H, *J* = 7.1 Hz, N**H**), 6.76 (d, 0.5H, *J* = 7.1 Hz, N**H**), 4.97 (q, 1H, *J* = 7.2 Hz, NHC**H**$CH_3$), 4.96-4.87 (m, 1H, C-3: $\mathbf{H}_\beta$), 3.85-3.80 (m, 1H, C-12: $\mathbf{H}_\beta$) 1.98-1.05 (b m, 24H, 6xC**H**, 9x$\mathbf{CH}_2$), 1.49 (d, 1.5H, *J* = 7.1 Hz, NHCHC$\mathbf{H}_3$), 1.48 (d, 1.5H, *J* = 7.1 Hz, NHCHC$\mathbf{H}_3$) 1.08 (d, 1.5H, *J* = 6.5 Hz, C-21: C$\mathbf{H}_3$), 1.07 (d, 1.5H, *J* = 6.5 Hz, C-21: C$\mathbf{H}_3$), 0.94 (t, 1.5H, *J* = 7.3 Hz, C-23: C$\mathbf{H}_3$), 0.93 (t, 1.5H, *J* = 7.3 Hz, C-23: C$\mathbf{H}_3$), 0.81 (s, 3H, C-19: C**H**3), 0.55 (s, 3H, C-18: C$\mathbf{H}_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 172.8, 166.7, 134.0, 131.5, 128.4, 127.0, 75.7, 73.1, 48.6, 48.1, 47.2, 46.3, 41.8, 36.6, 35.9, 34.7, 34.0, 33.6, 32.0, 28.5, 28.1, 27.3, 26.9, 26.3, 25.9, 23.5, 23.0, 18.6, 17.0, 12.7, 10.2; exact mass 523.3657 (523.3662 calcd for $C_{33}H_{49}NO_4$). Integration of the signals for the α-methyl protons in the $^1H$ NMR spectrum indicated a 50:50 mixture of diastereomers. A sample of **14** was hydrolysed and derivatised with Marfey's reagent[120] as described for **17**. HPLC analysis indicated that the hydrolysis product was a mixture of 48.2% L-alanine and 51.8% D-alanine.

**_N_-Benzoyl-D-alanine, 3α-(12α-Hydroxy)-24-nor-5β-cholanyl Ester (15)**

A solution of sterol **12** (0.174 g, 0.500 mmol) in dry $CH_2Cl_2$ (2 mL) was added to a mixture of *N*-benzoyl-D-alanine (0.193 g, 1.00 mmol), 1,1'-carbonyldiimidazole (0.162 g, 1.00 mmol), and allyl bromide (450 μL, 5.20 mmol) in dry $CH_2Cl_2$ (3.25 mL) followed by addition of dry $CH_3CN$ (5 mL). The mixture was stirred for 18 h at room temperature under argon, then the solvent was removed *in vacuo* and the residue was purified by silica MPLC (3.5 x 12 cm, 5% $CH_3CN/CHCl_3$) to give **15** (73.6 mg, 28%, colorless oil) as a

diastereomeric mixture: $^1$H NMR (360 MHz, $d_6$-benzene) δ 7.86-7.81 (m, 2H, *o*-Ar**H**), 7.14-7.04 (m, 3H, *m,p*-Ar**H**), 6.83 (b s, 1H, N**H**), 4.97 (q, 1H, *J* = 7.2 Hz, NHC**H**CH$_3$), 4.96-4.87 (m, 1H, C-3: **H**$_\beta$), 3.90-3.85 (m, 1H, C-12: **H**$_\beta$) 1.98-0.98 (b-m, 24H, 6xC**H**, 9xC**H**$_2$), 1.45 (d, 1.8H, *J* = 7.1 Hz, NHCHC**H**$_3$), 1.44 (d, 1.2H, *J* = 7.1 Hz, NHCHC**H**$_3$) 1.03 (d, 1.8H, *J* = 6.5 Hz, C-21: C**H**$_3$), 1.02 (d, 1.2H, *J* = 6.5 Hz, C-21: C**H**$_3$), 0.94 (t, 1.8H, *J* = 7.3 Hz, C-23: C**H**$_3$), 0.93 (t, 1.2H, *J* = 7.3 Hz, C-23: C**H**$_3$), 0.81 (s, 3H, C-19: C**H**$_3$), 0.60 (s, 3H, C-18: C**H**$_3$). $^{13}$C NMR (90 MHz, pyridine-$d_5$) δ 173.2, 167.9, 135.4, 131.6, 128.7, 128.2, 75.4, 72.4, 49.6, 48.4, 47.1, 47.0, 42.3, 37.6, 36.5, 35.2, 34.5, 34.0, 32.6, 29.8, 28.8, 28.1, 27.4, 26.9, 26.6, 24.3, 23.4, 17.9, 17.3, 13.1, 10.8. Integration of the signals for the α-methyl protons in the $^1$H NMR spectrum indicated a 60:40 mixture of diastereomers.

**_N_-Benzoyl-L-phenylalanine, 3α-(12α-Hydroxy)-24-nor-5β-cholanyl Ester (16)**

A solution of 1,1'-carbonyldiimidazole (0.265 g, 1.64 mmol) in $CHCl_3$ (5 mL, EtOH-free) under argon was added, via cannula, to *N*-benzoyl-L-phenylalanine (0.430 g, 1.60 mmol). The solution was stirred at room temperature for 1 h, and then the sterol **12** (0.349 g, 1.00 mmol) in $CHCl_3$ (5 mL) was added via cannula, followed by allyl bromide (570 μL, 6.60 mmol). The reaction mixture was stirred at room temperature for 1 h and then heated to reflux for 18 h. The solvent was removed *in vacuo* and the residue was redissolved in EtOAc (25 mL). This was washed with 0.5 N HCl (2x15 mL), 5% $NaHCO_3$ (2x15 mL), and brine (15 mL), and then dried over $Na_2SO_4$ and concentrated *in vacuo* to a yellow foam. Initial purification by flash chromatography (5x15 cm, 75-90% $CHCl_3$/hexanes) followed by silica MPLC (B-size column, 70-85% $CHCl_3$/hexanes) gave **16** (0.372 g, 62%, white foam) as a diastereomeric mixture: IR ($CHCl_3$ cast) 3455, 2936, 1733, 1653, 1521, 1214 cm$^{-1}$; $^1$H NMR (200 MHz, $CDCl_3$) δ 7.52-7.08 (m, 10H, Ar**H**), 6.58 (d, 1H, *J* = 7.7 Hz, N**H**), 5.05 (t, 0.5H, *J* = 5.5 Hz, NHC**H**CH$_2$Ar), 5.00 (t, 0.5H, *J* = 5.8 Hz,

NHC**H**$CH_2$Ar), 4.87-4.68 (m, 1H, C-3: **H**β), 4.00 (b s, 1H, C-12: **H**β), 3.25 (d, 1H, *J* = 5.7 Hz, CHC**H**$_2$Ar), 3.21 (d, 1H, *J* = 5.5 Hz, CHC**H**$_2$Ar), 1.98-0.98 (b m, 24H, 6xC**H**, 9xC**H**$_2$), 0.95 (d, 3H, *J* = 6.4 Hz, C-21: C**H**$_3$), 0.91 (s, 3H, C-19: C**H**$_3$), 0.82 (t, 3H, *J* = 7.3 Hz, C-23: C**H**$_3$), 0.67 (s, 3H, C-18: C**H**$_3$); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 171.1, 166.7, 135.9, 134.0, 131.6, 129.5, 128.5, 128.4, 127.0, 76.0, 73.2, 53.6, 48.2, 47.2, 46.4, 41.9, 37.9, 36.6, 35.9, 34.8, 34.1, 33.6, 32.0, 28.5, 28.1, 27.4, 26.9, 26.4, 26.0, 23.6, 23.0, 17.1, 12.7, 10.3; exact mass 599.3975 (599.3975 calcd for $C_{39}H_{53}NO_4$). Integration of the signals for the benzylic methylene protons in the $^1$H NMR spectrum indicated a 50:50 mixture of diastereomers.

**_N_-Benzoylalanine, 3α-(12α-Hydroxy)-24-nor-5β-cholanyl Ester (17a,b) from Methylation of 13.**

A stirred suspension of **13** (51.0 mg, 0.100 mmol) in a mixture of THF (3 mL) and HMPA (0.8 mL) under argon at -78 °C was treated dropwise with BuLi (450 μL of a 0.71 M solution in hexanes, 0.320 mmol) generating a strong orange color. The solution was stirred at -78 °C for 0.5 h then methyl iodide (6.85 μL, 0.110 mmol) was added with rapid loss of color. The reaction mixture was poured into saturated $NH_4Cl$ (6 mL) and the aqueous layer was extracted with ether (3 x 5 mL). The combined organic layers were washed with brine (2 x 7 mL) and dried over $Na_2SO_4$. Concentration *in vacuo* gave a yellow oil which was purified by flash chromatography (2 x 15 cm, 5% $CH_3CN/CHCl_3$) to afford **17a** (16.8 mg, 32%) as a clear oil: $^1$H NMR (200 MHz, $C_6D_6$) δ 7.82-7.74 (m, 2H, *o*-Ar**H**), 7.08-6.98 (m, 3H, *m*,*p*-Ar**H**), 6.88-6.63 (m, 1H, N**H**), 5.00-4.80 (m, 2H, C-3: **H**β, HNC**H**$CH_3$), 3.83-3.76 (m, 1H, C-12: **H**β), 1.98-0.70 (b m, 24H, 6xC**H**, 9xC**H**$_2$), 1.40 (d, 2.1H, *J* = 7.02 Hz, NHCHC**H**$_3$), 1.39 (d, 0.9H, *J* = 7.0 Hz, NHCHC**H**$_3$), 0.98 (d, 3H, *J* = 6.5 Hz, C-21: C**H**$_3$), 0.90 (t, 3H, *J* = 7.3 Hz, C-23: C**H**$_3$), 0.76 (s, 3H, C-19: C**H**$_3$), 0.55 (s, 3H, C-18: C**H**$_3$). $^{13}$C NMR (90 MHz, $CDCl_3$) δ 172.7, 166.5, 133.9, 131.5, 128.3, 127.1, 75.7, 73.0, 48.7, 48.2, 47.1, 46.4, 41.8,

36.6, 35.9, 34.6, 34.0, 33.5, 32.0, 28.4, 28.2, 27.2, 26.9, 26.5, 25.9, 23.3, 22.9, 18.7, 17.1, 12.6, 10.2. Integration of the signals for the α-methyl protons in the $^1$H NMR spectrum indicated a 70:30 mixture of diastereomers.

**Alternative procedure:** The sterol **13** (25.5 mg, 50.0 μmol) in THF (1.3 mL) was added via cannula to a stirred solution of LiHMDS (0.20 mmol) in THF (0.5 mL) at -78 °C under argon and stirring was continued for 0.5 h at which point methyl iodide (15.6 μL, 0.250 mmol) was added. Stirring was continued for a further 6.5 h and then the solution was poured into saturated $NH_4Cl$ (1 mL), and the aqueous layer was separated and extracted with $CHCl_3$ (2 x 2 mL). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo* to give a yellow oil. Purification by flash chromatography (1 x 6 cm, 5% $CH_3CN$/ $CHCl_3$) gave **17b** (19.7 mg, 75%) as a colorless oil. Spectral features were in agreement with those of **17a**. Integration of the alanine methyl doublets at δ 1.40 and 1.39 in the $^1$H NMR spectrum indicated a diastereomeric ratio of 60:40.

**Stereochemical analysis:** A solution of **17a** (10.8 mg, 20.5 μmol) in MeOH (0.5 mL) was added to a solution of lithium hydroxide monohydrate (11.0 mg, 262 μmol) in water (0.25 mL) and stirred vigorously for 5 h. The MeOH was removed *in vacuo* and the aqueous layer was initially extracted with EtOAc (2 x 1 mL) to remove the hydrolysed sterol. It was then acidified to pH 1 with 6 N HCl and extracted with EtOAc (3 x 1 mL). The latter organic layers were combined and concentrated *in vacuo*. The residue was dissolved in 6 N HCl (1 mL) and heated at reflux for 12 h. The solvent was removed *in vacuo* and the residue was redissolved in water (1 mL) and concentrated again to dryness. Compound **17b** was treated identically. The hydrolysed residues from **17a** and **17b** corresponded chromatographically and spectroscopically with an authentic sample of alanine.

The Marfey derivatives of these residues were prepared by the literature procedure.[120] Each residue was dissolved in water (400 μL) and 100 μL aliquots of each

solution (approx. 5 μmol) were added to solutions of *N*-1-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent, prepared according to the literature procedure,[120] 200 μL of a 1% solution in acetone, 2.0 mg, 7.2 μmol). The solutions were treated with 1 M $NaHCO_3$ (50 μL, 50 μmol) and stirred at 40 °C for 1 h. After cooling to room temperature, 2 N HCl (25 μL, 50 μmol) was added, and the solutions were concentrated to dryness *in vacuo*. The residues were each dissolved in DMSO (2 mL) to give 1.25 mM solutions. Standard solutions of the Marfey derivatives of authentic samples of L- and D-alanine were also prepared by the same procedure.

HPLC Analysis: The procedure of Marfey[120] was modified. Aliquots (10 μL) of the DMSO solutions of the Marfey derivatives were injected onto a Chemcosorb 5-ODS-UH reverse phase column (Chemcopak, 4.6 x 150 mm) on a Beckman System Gold HPLC (see General Experimental section) and eluted with 50 mM triethylammonium phosphate, pH 3.0, with a linear gradient of 20-40% $CH_3CN$ over 30 min with detection at 340 nm. Retention times were: L-alanine derivative, 10.4 min; D-alanine derivative, 14.3 min; hydrolysed reagent, 15.9 min. Relative detector response for the two derivatives (based on their reported $\varepsilon_M$ values[120]) was D-Ala : L-Ala = 1.03. The diastereomeric ratios, calculated from integration of the peak areas, were: 66.8% D-Ala, 33.2% L-Ala from **17a**; and 61.0% D-Ala, 39.0% L-Ala from **17b**.

**_N_-Benzoylglycine, 3α-(12α-Methoxymethyl ether)-24-nor-5β-cholanyl Ester (18)**

The general procedure of Fuji et al[198] was followed. Dimethoxymethane (1.00 mL, 11.5 mmol) and $P_2O_5$ (approx. 0.5 g) were added to a stirred solution of **13** (51.0 mg, 0.100 mmol) in dry $CHCl_3$ (1 mL) under argon, and the mixture was stirred for 2.5 h at room temperature. It was then poured into cold 10% $Na_2CO_3$ (2 mL), and the organic layer was washed with brine (2 mL) and dried over $Na_2SO_4$. Concentration *in vacuo* gave a colorless gum. Purification by preparative TLC (silica gel, 20 x 20 cm x 0.25 mm, 40%

EtOAc/hexanes) gave **18** (52.8 mg, 96%) as a colorless oil: IR ($CHCl_3$ cast) 3345, 2937, 1745, 1652, 1203, 1043 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 7.87-7.79 (m, 2H, *o*-Ar**H**), 7.55-7.40 (m, 3H, *m,p*-Ar**H**), 6.67 (b s, 1H, N**H**), 4.92-4.83 (m, 1H, C-3: **H**β), 4.72 (d, 1H, *J* = 6.6 Hz, $CH_3OC\mathbf{H}_aHOCH$), 4.69 (d, 1H, *J* = 6.7 Hz, $CH_3OCH\mathbf{H}_bOCH$), 4.20 (d, 2H, *J* = 4.9 Hz, $NHC\mathbf{H}_2CO_2$), 3.81 (t, 1H, *J* = 2.5 Hz, C-12: **H**β), 3.42 (s, 3H, $C\mathbf{H}_3OCH_2O$), 1.98-0.95 (b m, 24H, 6x**CH**, 9x$C\mathbf{H}_2$), 0.94 (s, 3H, C-19: $C\mathbf{H}_3$), 0.92 (d, 3H, *J* = 5.9 Hz, C-21: $C\mathbf{H}_3$), 0.87 (t, 3H, *J* = 7.1 Hz, C-23: $C\mathbf{H}_3$), 0.70 (s, 3H, C-18: $C\mathbf{H}_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 169.6, 167.3, 133.8, 131.7, 128.5, 127.0, 96.4, 80.8, 76.0, 56.2, 48.5, 46.4, 46.1, 42.1, 42.0, 37.3, 36.0, 34.8, 34.3, 34.0, 32.2, 28.3, 27.8, 27.1, 26.5, 26.1, 25.4, 23.9, 23.2, 17.4, 12.7, 10.4; MS (CI, $NH_3$) 571 ($MNH_4^+$), 554 ($MH^+$).

**_N_-Benzoylalanine, 3α-(12α-Methoxymethyl ether)-24-nor-5β-cholanyl Ester (19)**

Compound **19** was prepared by the same procedure as used for **18**. Thus dimethoxymethane (1.00 mL, 11.5 mmol) and phosphorus pentoxide (approx. 0.5 g) were added to **14** (52.4 mg, 0.100 mmol) in dry $CHCl_3$ (1 mL) and the mixture was stirred under argon for 0.5 h. Work-up as for **18** gave a yellow oil which was purified by flash chromatography (1.5 x 18 cm, $CHCl_3$) to give **19** (17.6 mg, 31%) as a colorless oil: IR ($CHCl_3$ cast) 3350, 2938, 1737, 1650, 1044 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 7.80-7.75 (m, 2H, *o*-Ar**H**), 7.50-7.37 (m, 3H, *m,p*-Ar**H**), 6.75 (b d, 1H, *J* = 7.0 Hz, 1H, N**H**), 4.87-4.63 (b m, 4H, C-3: **H**β, $OC\mathbf{H}_2OCH_3$, $NHC\mathbf{H}CH_3$), 3.75 (b s, 1H, C-12: **H**β), 3.40 (s, 1.5H, $OCH_2OC\mathbf{H}_3$), 3.39 (s, 1.5H, $OCH_2OC\mathbf{H}_3$), 1.98-0.95 (b m, 24H, 6x**CH**, 9x$C\mathbf{H}_2$), 1.48 (d, 1.5H, *J* = 7.1 Hz, $NHCHC\mathbf{H}_3$), 1.46 (d, 1.5H, *J* = 7.1 Hz, $NHCHC\mathbf{H}_3$), 0.91 (s, 3H, C-19: $C\mathbf{H}_3$), 0.89 (d, 3H, *J* = 6.2 Hz, C-21: $C\mathbf{H}_3$), 0.81 (t, 3H, *J* = 7.2 Hz, C-23: $C\mathbf{H}_3$), 0.67 (s, 3H, C-18: $C\mathbf{H}_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 172.8, 166.7, 134.0, 131.6, 128.5, 127.0, 96.4, 80.9, 75.8, 56.2, 48.6, 46.3, 46.1,

42.0, 37.2, 35.9, 34.8, 34.3, 34.0, 32.2, 28.3, 27.8, 27.1, 26.5, 26.4, 26.1, 25.5, 23.9, 23.2, 17.4, 12.7, 10.4; MS (CI, $NH_3$) 585 ($MNH_4^+$), 568 ($MH^+$). Anal. Calcd for $C_{35}H_{53}NO_5 \cdot H_2O$: C, 71.76; H, 9.46; N, 2.39. Found: C, 72.09; H, 9.61; N, 2.41. Integration of the α-methyl doublets in the $^1H$ NMR spectrum indicated a 50:50 mixture of diastereomers.

**_N_-Benzoylalanine, 3α-(12α-Methoxymethyl ether)-24-nor-5β-cholanyl Ester (20) from Methylation of 18**

A stirred solution of **18** (15.8 mg, 28.5 μmol) in dry THF (0.5 mL) under argon at -78 °C was treated dropwise with LiHMDS (0.25 mL of a 0.25 M solution in THF, 62.7 μmol). After stirring for 0.5 h, methyl iodide (20 μL, 321 μmol) was added, and stirring was continued for a further 0.5 h. Saturated $NH_4Cl$ (1 mL) was added, and the mixture was warmed to room temperature with vigorous stirring. The organic layer was washed with brine (3 mL) and dried over $NaSO_4$. Removal of the solvent *in vacuo* gave a yellow gum which was purified by flash silica chromatography (5 mm x 5 cm, $CHCl_3$) to give **20** (2.69 mg, 17%, oil) as a diastereomeric mixture: $^1H$ NMR (200 MHz, $CDCl_3$) δ 7.80-7.75 (m, 2H, *o*-Ar**H**), 7.53-7.36 (m, 3H, *m,p*-Ar**H**), 6.75 (b d, 1H, *J* = 7.0 Hz, 1H, N**H**), 4.87-4.63 (b m, 4H, C-3: **H**β, $OCH_2OCH_3$, $NHCHCH_3$), 3.80 (b s, 1H, C-12: **H**β), 3.41 (s, 1.2H, $OCH_2OCH_3$), 3.39 (s, 1.8H, $OCH_2OCH_3$), 1.98-0.95 (b m, 24H, 6x**CH**, 9x$CH_2$), 1.49 (d, 1.2H, *J* = 7.1 Hz, $NHCHCH_3$), 1.47 (d, 1.8H, *J* = 7.1 Hz, $NHCHCH_3$), 0.91 (s, 3H, C-19: $CH_3$), 0.89 (d, 3H, *J* = 6.2 Hz, C-21: $CH_3$), 0.81 (t, 3H, *J* = 7.2 Hz, C-23: $CH_3$), 0.67 (s, 3H, C-18: $CH_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 173.0, 166.9, 131.7, 128.5, 127.0, 96.5, 80.7, 75.8, 56.2, 48.6, 46.4, 46.1, 42.1, 37.2, 35.8, 34.8, 34.3, 33.9, 32.2, 28.5, 27.8, 27.2, 26.5, 26.4, 26.0, 25.5, 23.9, 23.2, 17.4, 12.8, 10.4. Integration of the α-methyl doublets in the $^1H$ NMR spectrum indicated a 40:60 mixture of diastereomers.

**_N_-Benzoyl-α'-methyl-phenylalanine, 3α-(12α-Hydroxy)-24-nor-5β-cholanyl Ester (21) from Benzylation of 14**

A stirred solution of **14** (26.2 mg, 50.0 μmol) in dry THF (0.5 mL) under argon at -70 °C was treated dropwise with LDA (110 μL of a 1.55 M solution in THF, 170. μmol). Stirring of the orange solution was continued for 15 min. Benzyl bromide (50 μL, 420. μmol) was added and stirring was continued at -70 °C for 1.5 h. Saturated $NH_4Cl$ (1 mL) was added, and the solution was warmed to room temperature. The mixture was extracted with EtOAc (2 x 2 mL), and the combined organic layers were dried over $Na_2SO_4$ and concentrated *in vacuo* to a colorless oil. Purification by preparative TLC (silica gel, 20 x 20 cm x 0.25 mm, 3% $CH_3CN/CHCl_3$) gave **21** (26.8 mg, 87%, oil) as a mixture of diastereomers: IR ($CHCl_3$ cast) 3405, 2937, 1724, 1653, 1519, 1450, 1213 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$) δ 7.69-7.08 (m, 10H, Ar**H**), 6.80 (d, 1H, *J* = 9.5 Hz, N**H**), 4.86-4.75 (m, 1H, C-3: $\mathbf{H}_\beta$), 4.03 (b s, 1H, C-12: $\mathbf{H}_\beta$), 3.76 (d, 0.55H, *J* = 13.5 Hz, C$\mathbf{H}_a$HAr), 3.74 (d, 0.45H, *J* = 13.5 Hz, C$\mathbf{H}_{a'}$HAr), 3.28 (d, 0.45H, *J* = 13.5 Hz, CH$\mathbf{H}_{b'}$Ar), 3.27 (d, 0.55H, *J* = 13.5 Hz, CH$\mathbf{H}_b$Ar), 1.92-0.98 (b m, 24H, 6xC**H**, 9xC$\mathbf{H}_2$), 1.80 (s, 1.65H, C$\mathbf{H}_3$CCH$_2$Ar), 1.78 (s, 1.35H, C$\mathbf{H}_3$CCH$_2$Ar), 0.97 (d, 3H *J* = 6.6 Hz, C-21: C$\mathbf{H}_3$), 0.95 (s, 3H, C-19: C$\mathbf{H}_3$), 0.84 (t, 3H, *J* = 7.3 Hz, C-23: C$\mathbf{H}_3$), 0.70 (s, 3H, C-18: C$\mathbf{H}_3$); $^{13}C$ NMR (90 MHz, $CDCl_3$) δ 173.6, 166.8, 136.5, 135.3, 131.3, 130.0, 128.5, 128.1, 126.8, 76.3, 73.3, 61.5, 48.4, 47.4, 46.5, 42.0, 40.9, 36.6, 36.0, 34.9, 32.1, 28.6, 28.2, 27.4, 26.9, 26.6, 26.0, 23.6, 23.1, 17.2, 12.7, 10.3; exact mass 613.4140 (613.4131 calcd for $C_{40}H_{55}NO_4$); MS (CI, $NH_3$) 614 (100%, $MH^+$). Integration of the signals for the benzylic methylene and α-methyl protons in the $^1H$ NMR spectrum indicated a 55:45 mixture of diastereomers.

**_N_-Benzoyl-α'-methyl-phenylalanine, 3α-(12α-Methoxymethyl ether)-24-nor-5β-cholanyl Ester (22) from Benzylation of 19**

A stirred solution of **19** (25.0 mg, 44.0 μmol) in dry THF (1 mL) under argon at -70 °C was treated dropwise with LDA (100. μL of a 1.00 M solution in THF, 100 μmol). After 40 min, benzyl bromide ( 50 μL, 420 μmol) was added. Stirring was continued at -70 °C for 1 h, and then the mixture was slowly warmed to room temperature over 2 h. Saturated $NH_4Cl$ (0.5 mL) was added, and the mixture was extracted with EtOAc (3 x 1 mL). The combined organic layers were dried over $Na_2SO_4$ and concentrated *in vacuo* to a yellow oil (73.5 mg). Purification by flash chromatography (1 x 15 cm, 2.5-10% EtOAc/hexanes) afforded **22** (17.2 mg, 59%) as a clear oil: IR ($CHCl_3$ cast) 3410, 3320, 2938, 1728, 1663, 1214, 756 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 7.70-7.05 (b m, 10H, Ar**H**), 6.86 (d, 1H, $J$ = 6.8 Hz, N**H**), 4.91-4.64 (m, 3H, C-3: $\mathbf{H}_\beta$, $CH_3OC\mathbf{H}_2O$), 3.83 (b s, 1H, C-12: $\mathbf{H}_\beta$), 3.77 (d, 0.5H, $J$ = 13.5 Hz, $C\mathbf{H}_a$HAr), 3.75 (d, 0.5H, $J$ = 13.5 Hz, $C\mathbf{H}_a$HAr), 3.43 (s, 1.5H, $C\mathbf{H}_3OCH_2O$), 3.40 (s, 1.5H, $C\mathbf{H}_3OCH_2O$), 3.28 (d, 0.5H, $J$ = 13.5 Hz, $CH\mathbf{H}_b$Ar), 3.26 (d, 0.5H, $J$ = 13.5 Hz, $CH\mathbf{H}_b$Ar), 2.02-0.98 (b m, 24H, 6x**CH**, 9x$C\mathbf{H}_2$), 1.80 (s, 1.5H, $C\mathbf{H}_3CCH_2Ar$), 1.77 (s, 1.5H, $C\mathbf{H}_3CCH_2Ar$), 0.96 (s, 3H, C-19: $C\mathbf{H}_3$), 0.94 (d, 3H $J$ = 6.6 Hz, C-21: $C\mathbf{H}_3$), 0.84 (t, 3H, $J$ = 7.3 Hz, C-23: $C\mathbf{H}_3$), 0.71 (s, 3H, C-18: $C\mathbf{H}_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 136.9, 135.8, 131.4, 130.0, 129.9, 128.5, 128.1, 126.8, 96.4, 81.0, 76.3, 61.3, 56.2, 48.6, 46.4, 46.2, 42.0, 40.9, 37.2, 36.0, 35.0, 34.3, 34.1, 32.5, 28.3, 27.8, 27.2, 26.3, 26.1, 25.5, 23.9, 23.6, 23.2, 17.4, 12.7, 10.4; exact mass 597.4154 (597.4182 calcd for $C_{40}H_{55}NO_3$, $M^+$-$C_2H_4O_2$); MS (CI, $NH_3$) 658 ($MH^+$). Integration of the signals for the benzylic methylene and α-methyl protons in the $^1H$ NMR spectrum indicated a 50:50 mixture of diastereomers.

**_N_-Benzoyl-α'-methyl-phenylalanine, 3α-(12α-Hydroxy)-24-nor-5β-cholanyl Ester (23) from Methylation of 16**

A stirred solution of **16** (30.0 mg, 50.0 μmol) in dry THF (0.5 mL) under argon at -78 °C was treated dropwise with LDA (10.6 μL of a 1.56 M solution in THF, 165 μmol). After 15 min, methyl iodide ( 50 μL, 803 μmol) was added and the reaction mixture was stirred at -78 °C for 1.5 h. Work up and purification as for **21** gave **23** (29.4 mg, 96%, oil) as a diastereomeric mixture. Spectral characteristics were similar to those of **21**. Integration of the benzylic methylene and α-methyl signals in the $^1$H NMR spectrum indicated a 65:35 ratio of diastereomers, with the same isomer predominating as in **21**.

**Bis-[(1*R*, 2*S*)- *trans*-2-Phenylcyclohexyl] 1,7-Heptandioate (24)**

A solution of pimeloyl dichloride (1.46 g, 7.41 mmol, prepared from pimelic acid by reaction with thionyl chloride according to the method of Cason and Reist[201]) in dry ether (3 mL) was added dropwise to a solution of alcohol **28** (2.10 g, 11.9 mmol) in ether (5 mL). The solution was stirred for 2 h, and then distilled pyridine (1.00 mL, 12.4 mmol) was added, thereby immediately generating a white precipitate. The mixture was stirred for a further 16 h and then poured into saturated aqueous $K_2CO_3$ (5 mL). The aqueous layer was extracted with ether (5 mL). The combined organic layers were washed with water (5 mL), dried over $MgSO_4$, and concentrated *in vacuo* giving a clear viscous oil (2.27 g). Purification by flash chromatography (5 x 20 cm, 18% EtOAc/hexanes) followed by recrystallisation from EtOH afforded **24** (1.43 g, 50%) as large white crystals: mp 58.0-58.7 °C; IR ($CHCl_3$ cast) 2932, 2859, 1730, 1450, 1175, 700 cm$^{-1}$; $^1$H NMR (300 MHz, $CDCl_3$) δ 7.30-7.13 (m, 10H, Ar**H**), 5.03 (ddd, 2H, $J$ = 10.6, 10.6, 4.4 Hz, 2 C**H**OR), 2.70 (ddd, 2H, $J$ = 12.0, 11.1, 3.7 Hz, 2 C**H**Ar) 2.18-2.05 (m, 2H, 2 C**H**$_a$HCHOR), 1.98-1.74 (m, 10H, 2 CH**H**$_b$CHOR, 2 C**H**$_2$CHAr, 2 C**H**$_2$CO$_2$), 1.66-1.27 (m, 8H, 2 C**H**$_2$C**H**$_2$CH$_2$CHOR), 1.20-1.03 (tt, 4H, $J$ = 7.7, 7.5 Hz, 2 x C**H**$_2$CH$_2$CO$_2$), 0.75-0.60 (m, 2H, C**H**$_2$CH$_2$CH$_2$CO$_2$); $^{13}$C NMR (75.5 MHz, $CDCl_3$) δ 172.7 (**C**O$_2$R), 143.1

(Ar), 128.2 (Ar), 127.4 (Ar), 126.3 (Ar), 75.5 (**C**HOR), 49.8 (**C**HAr), 34.1 (**C**$H_2CO_2R$), 33.9 (**C**$H_2$CHOR), 32.3 (**C**$H_2$CHAr), 27.8 (**C**$H_2CH_2CH_2CO_2R$), 25.8 (**C**$H_2$), 24.7 (**C**$H_2$), 24.4 (**C**$H_2CH_2CO_2R$); MS (CI, $NH_3$) 477.40 ($MH^+$). Anal. Calcd for $C_{31}H_{40}O_4$: C, 78.12; H, 8.46. Found: C, 77.96; H, 8.66.

**2,4,6-Triisopropylbenzenesulfonyl Azide (Trisyl Azide, 25)**

The compound was prepared according to the method used by Leffler and Tsuno[131] for the preparation of similar compounds. A suspension of 2,4,6-triisopropylbenzenesulfonyl chloride (97%, 9.57 g, 30.7 mmol) in acetone (20 mL) was added dropwise to a mixture of $NaN_3$ (2.68 g, 40.8 mmol) in water (7 mL) and acetone (10 mL) at 0 °C. After stirring at 0 °C for 0.5 h the mixture was warmed to room temperature and stirred for 3.5 h. Water (10 mL) was added and the mixture was extracted with ether (2 x 10 mL). The combined organic fractions were washed with ice/water (4 x 10 mL), dried over $NaSO_4$, and concentrated *in vacuo*, affording 9.40 g of a yellow solid. Filtration through a short column of silica (20% $CH_2Cl_2$/hexanes) followed by recrystallization from distilled hexanes gave **25** (8.11 g, 85%) as fine white crystals: mp 42.3-43.0 °C (lit.[200] mp 41-43 °C); IR ($CHCl_3$ cast) 2941, 2121 ($N_3$), 1195, 1167 ($SO_2$), 590 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$) δ 7.20 (s, 2H, Ar**H**), 4.04 (septet, 2H, $J$ = 6.7 Hz, *o*-C**H**$(CH_3)_2$), 2.91 (septet, 1H, $J$ = 6.9 Hz, *p*-C**H**$(CH_3)_2$), 1.27 (d, 12H, $J$ = 6.7 Hz, *o*-CH(C**H**$_3)_2$), 1.25 (d, 6H, $J$ = 6.9 Hz, *p*-CH(C**H**$_3)_2$); $^{13}C$ NMR (50 MHz, $CDCl_3$) δ 154.6, (**C**-$SO_2N_3$), 151.0 (*o*-**C**CH$(CH_3)_2$), 132.1 (*p*-**C**CH$(CH_3)_2$), 124.1 (*m*-Ar**C**), 34.3 (*p*-**C**H$(CH_3)_2$), 29.7 (*o*-CH(**C**H$_3)_2$), 23.4 (*p*-CH(**C**H$_3)_2$); exact mass 309.1510 (309.1511 calcd for $C_{15}H_{23}N_3O_2S$).

***trans*-2-Phenylcyclohexanol (26)**

The procedure of Whitesell and Lawrence was followed.[130] Bromobenzene (15 mL) was added to magnesium turnings (35.3 g, 1.45 mol) in THF (500 mL) under argon. Upon

commencement of reflux the dropwise addition of bromobenzene (155 mL total, 1.47 mol) was continued for 0.5 h. The reaction mixture was then heated to reflux for 1 h, cooled to room temperature and diluted with THF (900 mL). The solution was further cooled to -15 °C ($CCl_4$, $CO_2$), purified CuCl (6.59 g, 67 mmol) was added, and the mixture was stirred for 10 min. A solution of cyclohexene oxide (103 mL, 1.00 mol) in THF (100 mL) was added dropwise over 45 min with cooling to keep the reaction temperature below 10 °C. The mixture was then stirred for 2.5 h at -5 °C. The gray suspension was poured into saturated $(NH_4)_2SO_4$ (500 mL) to give a bright blue biphasic solution and a blue-gray precipitate. The THF solution was separated and washed with saturated $(NH_4)_2SO_4$ (3 x 250 mL) until the aqueous layer was colorless. The combined aqueous layers were extracted with ether (3 x 100 mL), and the precipitate was washed with ether (150 mL). The combined organic layers were dried over $MgSO_4$, and the solvent was removed *in vacuo* to give 192 g of a golden oil which crystallised on standing. Recrystallization from pentane gave **26** (146 g, 83%) as white crystals: mp 55.3-57.5 °C (lit.[130] mp 56.5-57.0 °C; lit.[199] mp 57-58 °C); IR ($CHCl_3$ cast) 3272, 2930, 2854, 1444, 1050, 695 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$) δ 7.38-7.23 (m, 5H, Ar**H**), 3.68 (ddd, 1H, $J$ = 10.1, 10.1, 4.3 Hz, C**H**OH), 2.45 (ddd, 1H, $J$ = 12.4, 10.0, 3.5 Hz, C**H**Ar), 2.16-2.10 (m, 1H, $CH_aHCHOH$), 1.92-1.85 (m, 2H, $CHH_bCHOH$, $CH_aHCHAr$), 1.81 - 1.75 (m, 1H, $CH_bHCHAr$), 1.58-1.25 (m, 5H, $CH_2CH_2$, OH); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 143.3 (Ar), 128.7 (Ar), 127.9 (Ar), 126.7 (Ar), 74.3 (**C**HOH), 53.2 (**C**HAr), 34.4 ($CH_2CHOH$), 33.3 ($CH_2CHAr$), 26.0 ($CH_2$), 25.0 ($CH_2$); exact mass 176.1197 (176.1201 calcd for $C_{12}H_{16}O$).

**(1*R*, 2*S*) and (1*S*, 2*R*)-*trans*-2-Phenylcyclohexyl Acetate (27)**

The literature preparation of this compound by Whitesell and Lawrence was followed.[130] Compound **26** (88.0 g, 0.500 mol) in $CH_2Cl_2$ (125 mL) was added dropwise to a solution of *N,N*-4-dimethylaminopyridine (2.10 g, 17.2 mmol) and pyridine (90.0 mL, 1.10 mol)

in $CH_2Cl_2$ (300 mL) over 0.5 h. This was followed by a solution of acetic anhydride (94.1 mL, 1.00 mol) in $CH_2Cl_2$ (75 mL) added dropwise over 1.5 h. The mixture was stirred for a further 2 h, and then poured into a mixture of 5.7 N HCl (300 mL), ice (400 mL), and ether (750 mL). The organic phase was washed with 2 N HCl (4 x 150 mL). The combined aqueous layers were extracted with ether (2 x 350 mL). The combined organic fractions were washed with saturated $NaHCO_3$ (2 x 200 mL) and dried over $MgSO_4$. Concentration *in vacuo* gave a yellow oil which was vacuum distilled (0.35 mm Hg) to afford **27** (105 g, 96%) as a colorless oil: bp 104-105 °C at 0.35 mm Hg (lit.[129] bp 104-105 °C at 0.4 mm Hg); IR ($CHCl_3$ cast) 3028, 2935, 2858, 1737, 1495, 1452, 1242, 1037 $cm^{-1}$; $^1H$ NMR ( 200 MHz, $CDCl_3$) δ 7.32-7.14 (m, 5H, Ar**H**), 4.97 (ddd, 1H, $J$ = 10.6, 10.6, 4.4 Hz, C**H**OR), 2.63 (ddd, 1H, $J$ = 12.2, 10.6, 3.9 Hz, C**H**Ar), 2.20-2.08 (m, 1H, C$\mathbf{H}_a$HCHOR), 2.03-1.73 (m, 3H, C$\mathbf{H}_2$CHAr, CH$\mathbf{H}_b$CHOR), 1.78 (s, 3H, C$\mathbf{H}_3$), 1.61-1.30 (m, 4H, C$\mathbf{H}_2$C$\mathbf{H}_2$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 170.1 (**C**$O_2$R), 143.0 (Ar), 128.1 (Ar), 127.4 (Ar), 126.3 (Ar), 75.8 (**C**HOR), 49.7 (**C**HAr), 33.7 (**C**$H_2$CHOR), 32.3 (**C**$H_2$CHAr), 25.7 (**C**$H_2$), 24.7 (**C**$H_2$), 20.8 (**C**$H_3$); exact mass 218.1304 (218.1307 calcd for $C_{14}H_{18}O_2$).

**Enzymatic Resolution of (1*R*, 2*S*) and (1*S*, 2*R*)-*trans*-2-Phenylcyclohexyl Acetate (27) to (1*R*, 2*S*)-(-)-*trans*-2-Phenylcyclohexanol (28) and (1*S*, 2*R*)-(+)-*trans*-2-Phenylcyclohexyl Acetate (29)**

The procedure of Whitesell and Lawrence[130] as modified by Lane-Bell[129] was followed. Thus hydrolysis of the acetate **27** (83.2 g, 381 mmol) in acetone (446 mL) and 0.5 M $KH_2PO_4/K_2HPO_4$ buffer pH 8.0 (3.25 L) with pig liver acetone powder (PLAP) (17.4 g) was monitored by periodic removal of 1 mL aliquots which were acidified to pH 1 and extracted with $CH_2Cl_2$. These organic aliquots were dried over $Na_2SO_4$ and concentrated *in vacuo*. The residue was dissolved in $CDCl_3$. Integration of the $^1H$ NMR (80 MHz) spectrum and comparison of the CHOR versus C**H**OH signals indicated the extent of

reaction. The final ratio obtained was approximately 45:55 (alcohol:acetate ) after 15 days. Work up afforded 75.1 g of a brown oil. Purification by flash chromatography (10 x 30 cm, 3-30% EtOAc /hexanes) yielded 28.0 g (93%) of the alcohol **28** and 43.6 g (95%) of the acetate **29**. Recrystallization of **28** from distilled pentane afforded 26.2g (87%) of optically pure (-)-alcohol: mp 65.2-66.1 °C (lit.[130] mp 64-65 °C) $[\alpha]_D$= -58.8° (c = 4.16, MeOH) (lit.[130] $[\alpha]_D^{27}$= -58.4 ° (c = 10.0, MeOH)); IR ($CHCl_3$ cast) 3290, 2937, 2927, 2920, 1445, 1061, 1051 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$ ) δ 7.42-7.22 (m, 5H, Ar**H**), 3.65 (m, 1H, C**H**OH), 2.42 (ddd, 1H, *J* = 12.2, 9.8, 3.7 Hz, C**H**Ar), 2.16-2.09 (m, 1H, C**H**$_a$HCHOH), 1.92-1.83 (m, 2H, C**H**$_a$HCHAr and CH**H**$_b$CHOH), 1.83-1.72 (m, 1H, CH**H**$_b$CHAr), 1.63-1.26 (m, 5H, C**H**$_2$C**H**$_2$, O**H**); exact mass 176.1201 (176.1201 calcd for $C_{12}H_{16}O$). Anal. Calcd.for $C_{12}H_{16}O$: C, 81.77; H, 9.15. Found: C, 81.73; H, 9.27.

For **29**: IR ($CHCl_3$ cast) 2933, 1736, 1372, 1241 $cm^{-1}$ ; $^1H$ NMR (360 MHz, $CDCl_3$) δ 7.37-7.26 (m, 5H, Ar**H**), 5.06 (ddd, 1H, *J*= 10.5, 10.5, 4.5 Hz, C**H**OR), 2.74 (ddd, 1H, *J* = 12.8, 10.5, 3.8 Hz,C**H**Ar), 2.22- 2.19 (m, 1H, C**H**$_a$HCHOR), 2.04-1.82 (m, 3H, CH**H**$_b$CHOR, C**H**$_2$CHAr), 1.84 (s, 3H, C**H**$_3$), 1.67-1.35 (m, 4H, C**H**$_2$C**H**$_2$); exact mass 218.1302 (218.1307 calcd for $C_{14}H_{18}O_2$).

**(1*S*, 2*R*)-(+)-*trans*-2-Phenylcyclohexanol (30)**

The preparation of Whitesell and Lawrence[130] was followed. A solution of **29** (0.500 g, 2.29 mmol) and potassium hydroxide (0.91 g, 13.8 mmol) in MeOH (8 mL) was stirred at 50 °C for 1.5 h. The mixture was concentrated *in vacuo* and the residue was partitioned between water (10 mL) and EtOAc (10 mL). The aqueous phase was further extracted with EtOAc (3 x 5 mL), and the combined organic extracts were dried over $Na_2SO_4$. Concentration *in vacuo* yielded a yellow solid which was recrystallised from distilled pentane to afford alcohol **30** (180 mg, 45%) as feathery white crystals with 98.7% e.e.: mp 64.0-64.5 °C (lit.[130] mp 64-65 °C); $[\alpha]_D^{23}$ = +57.55° (c = 8.69, MeOH) (lit.[130] $[\alpha]_D^{23}$ = +58.3° (c=10.0, MeOH)); IR ($CHCl_3$ cast) 3290, 2937, 2923, 2920, 1445,

1061, 1051 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$) δ 7.38-7.23 (m, 5H, Ar**H**), 3.68 (ddd, 1H, *J* = 10.1, 10.1, 4.3 Hz, **CH**OH), 2.45 (ddd, 1H, *J* = 12.4, 10.0, 3.5 Hz, **CH**Ar), 2.16-2.10 (m, 1H, $CH_aHCHOH$), 1.92-1.85 (m, 2H, $CHH_bCHOH$, $CH_aHCHAr$), 1.81-1.75 (m, 1H, $CH_bHCHAr$), 1.58-1.25 (m, 5H, $CH_2CH_2$, OH); exact mass 176.1198 (176.1201 calcd for $C_{12}H_{16}O$). Anal. Calcd for $C_{12}H_{16}O$: C, 81.77; H, 9.15. Found: C, 81.90; H, 9.38.

**Attempted Azidation of Bis-[(1*R*, 2*S*)-*trans*-2-Phenylcyclohexyl] 1,7-Heptandioate (24)**

The general procedure of Evans and Britton [9] was followed. A dispersion of KH in oil (0.310 g, 35% w/w, 2.70 mmol) was washed with dry THF (3 x 3 mL) under argon to remove the oil. THF (15 mL) and 1,1,1,3,3,3-hexamethyldisilazane (0.38 g, 2.35 mmol) were added, and the mixture was stirred vigorously for 2 h and then allowed to settle for 0.5 h. The KHMDS solution (2.35 mmol) was added via cannula to a stirred solution of **18** (0.950 g, 1.99 mmol) under argon in THF (1 mL) at -78 °C. The mixture was stirred for 0.5 h and then a precooled solution (-78 °C) of trisyl azide **25** (0.740 mg, 2.40 mmol) in THF (3 mL) was added via cannula. The mixture was stirred for 2 min and glacial acetic acid (0.5 mL) was added. The cooling bath was removed, and the mixture was stirred at room temperature for 12 h. The mixture was partitioned between $CH_2Cl_2$ (40 mL) and dilute brine (40 mL). The aqueous phase was extracted with $CH_2Cl_2$ (3 x 20 mL), and the combined organic phases were washed with saturated $NaHCO_3$, dried over $MgSO_4$, and evaporated *in vacuo* to give a colorless oil (1.86 g). TLC (silica, 10% EtOAc/hexanes) showed a mixture of 4 major spots with $R_f$ values of: 0.54, 0.42, 0.34, and 0.18. Purification by flash silica chromatography (5 x 20 cm, 35-100% $CH_2Cl_2$/hexanes then 40% EtOAc/$CH_2Cl_2$) provided (in order of elution): 0.536 g of a yellow oil ($R_f$ = 0.54) which was recrystallised from hexanes to give trisyl azide (**25**) as white crystals (0.33 g, 45% recovery); 0.163 g of a white solid ($R_f$ = 0.42, **32**, 19%); 87.2 mg of a yellow oil

(Rf = 0.34, **31**, still a mixture by TLC); and 0.683 g of a colorless oil ($R_f$ = 0.18, diester **24**, 72% recovery).

Compound **31** was further purified by preparative TLC (silica gel, run in $CH_2Cl_2$, air-dried and repeated). It was identified as (1*R*, 2*S*)-*trans*-2-phenylcyclohexyl 2-cyclohexanone-carboxylate: IR ($CHCl_3$ cast) 2948, 2858, 2360, 1648, 1612, 1219 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$) δ 12.06 (s, 0.35H, enol O**H**); 7.26-7.15 (m, 5H, Ar**H**); 5.12-5.00 (m, 0.5H, C**H**OR); 4.93 (ddd, 0.5H, *J* = 10.6, 10.6, 4.3 Hz, C**H**OR); 3.08 (t, 0.25H, *J* = 5.7 Hz, $CH_2CHCO_2R$), 3.04 (t, 0.25H, *J* = 5.7 Hz, $CH_2CHCO_2R$); 2.74-2.61 (m, 1H, C**H**Ar), 2.26-1.30 (b m, 16H, 8 x $CH_2$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 206.4 (**C**(O)CHCO2R), 171.9, 171.5 (2 x $CO_2R$), 169.3 (enol: $CO_2R$), 143.0 (Ar), 128.2 (Ar), 127.5 (Ar), 126.3 (Ar), 97.8 (enol: C(O)$CCO_2R$), 76.9, 76.6, 76.3 (3 x CHOR), 56.8, 56.4 (2 x C(O)$CHCO_2R$), 49.7, 49.6 (CHAr), 41.1, 40.4 (2 x $CH_2C(O)$), 34.3, 33.9, 33.5 (3 x $CH_2CHOR$), 32.4, 32.1, 32.0 (3 x $CH_2CHAr$), 29.7, 29.6, 28.9 (3 x $CH_2CH_2CHCO_2R$), 26.9, 26.8 (2 x $CH_2CH_2C(O)$), 25.8, 25.7 (2 x $CH_2$), 24.7 ($CH_2$), 22.2, 22.1, 21.7 ($CH_2CH_2CHCO_2R$); exact mass 300.1731 (300.1725 calcd for $C_{19}H_{24}O_3$).

Compound **32** was identified as (1*R*, 2*S*)-*trans*-2-phenylcyclohexyl 2,4,6-triisopropylbenzenesulfonate: IR ($CHCl_3$ cast) 2958, 2935, 1178 ($SO_2$), 939, 875, 832, 795 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 7.22-7.12 (m, 5H, Ph**H**); 7.05 (s, 2H, Ar**H**); 5.00 (ddd, 1H, *J* = 10.7, 10.7, 4.5 Hz, C**H**OR), 3.88 (septet, 2H, *J* = 6.7 Hz, *o*-C**H**$(CH_3)_2$); 2.85 (septet, 1H, *J* = 7.0 Hz, *p*-C**H**$(CH_3)_2$), 2.70 (ddd, 1H, *J* = 11.3, 10.9, 3.9 Hz, C**H** Ph); 2.44-2.34, (m, 1H, C**H**$_a$HCHOR), 2.04-1.28, (b m, 7H, $CH_2CH_2CH_2CH$**H**$_b$CHOR), 1.22 (d, 6H, *J* = 7.0 Hz, *p*-CH(C**H**$_3)_2$); 1.12 (d, 12H, *J* = 6.7 Hz, *o*-CH(C**H**$_3)_2$); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) δ 152.9 (**C**$SO_2$), 150.0 (*o*-**C**$CH(CH_3)_2$), 142.2 (Ph**C**), 131.8 (*p*-**C**$CH(CH_3)_2$), 128.3 (Ar), 127.5 (Ar), 126.5 (Ar), 123.3 (Ar), 83.5 (**C**$HOSO_2Ar$), 50.1 (**C**HAr), 34.9 ($CH_2$), 34.2 (*p*-**C**$H(CH_3)_2$), 33.9 (**C**H2), 29.5 (*o*-**C**$H(CH_3)_2$), 25.5 ($CH_2$), 24.8 ($CH_2$), 24.7 (CH(**C**$H_3)_2$), 23.6

($CH(CH_3)_2$); exact mass 442.2526 (442.2542 calcd for $C_{27}H_{38}O_3S$). Anal. Calcd for $C_{27}H_{38}O_3S$: C, 73.26; H, 8.65, S, 7.24. Found: C, 73.03; H, 8.86; S, 7.40.

**(2*R*, 5*R*)-1-Benzoyl-5-(3'-bromopropyl)-2-*tert*-butyl-3-methyl-4-imidazolidinone (34).**

(2*R*)-1-Benzoyl-2-*tert*-butyl-3-methyl-4-imidazolidinone (0.521 g, 2.00 mmol) in dry THF (4 mL) and DMPU (0.6 mL) were added via cannula to a stirred solution of LDA [prepared from diisopropylamine (308 μL, 2.20 mmol) and n-BuLi (1.44 mL,1.53 M in hexane, 2.20 mmol)] in THF (2 mL) at -70 °C under argon. The mixture was stirred for 40 min, and 1,3-dibromopropane (271 μL, 2.67 mmol, filtered through basic alumina) was added to the deep red solution, followed by a further portion of DMPU (0.9 mL) for complete dissolution. The solution was slowly warmed to -15 °C over 2.5 h. Saturated $NH_4Cl$ (1 mL) was added and the mixture was warmed to room temperature. The THF was removed *in vacuo* and the residue was partitioned into ether (30 mL) and extracted with 2 N citric acid (2 x 10 mL), which was then back-extracted with ether (3 x 20 mL). The combined organic phases were washed with saturated $NaHCO_3$ (15 mL) and brine (15 mL) and dried over $Na_2SO_4$. Concentration *in vacuo* gave a yellow oil which was purified by flash chromatography (4 x 15 cm, $CHCl_3$) to afford **34** (621 mg, 81%) as an amorphous solid: IR ($CH_2Cl_2$ cast) 2970, 1707, 1640, 1360, 1260 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 7.70-7.40 (m, 5H, Ar**H**), 5.65 (s, 1H, $(CH_3)_3CHN$), 4.38 (b d, 1H, $J$ = 4 Hz, C(O)C**H**NC(O)), 3.10 (s, 3H, $NCH_3$), 3.15-2.92 (m, 2H, $CH_2Br$), 1.92-0.92 (m, 4H, $CH_2CH_2CH_2Br$), 1.08 (s, 9H, $(CH_3)_3C$); $^{13}C$ NMR (90 MHz, $CDCl_3$) δ 170.9 (**N**C(O)CHN), 136.2 (Ar), 129.0 (Ar), 128.7 (Ar), 127.3 (Ar), 80.1 (N**C**HN), 60.6 (N**C**HC(O)N), 40.9 ($(CH_3)_3C$), 31.9 ($CH_2$), 31.8 ($NCH_3$), 29.8 ($CH_2$), 25.9 ($CH_2$), 26.3 ($(CH_3)_3C$); MS (CI, $NH_3$) 383 (97%, $MH^+(^{81}Br)$), 381 (100%, $MH^+(^{79}Br)$). Anal. Calcd for $C_{18}H_{25}BrN_2O_2$: C, 56.70; H, 6.61; N, 7.35. Found: C, 56.72; H, 6.50; N, 7.30.

**(2*R*, 5*R*)-1-Benzoyl-2-*tert*-butyl-5-(3'-iodopropyl)-3-methyl-4-imidazolidinone (35).**

Compound **35** was prepared by a procedure analogous to that for the preparation of **34**. Thus addition of (2*R*)-1-benzoyl-2-*tert*-butyl-3-methyl-4-imidazolidinone (0.389 g, 1.49 mmol) in THF (4 mL) and DMPU (1 mL) to a solution of LDA (1.5 mmol) in THF (2 mL), was followed after 1 h by addition of 1,3-diiodopropane (517 μL, 4.50 mmol, filtered through basic alumina) to give, after work-up and purification (flash chromatography, 3 x 5 cm, 30% EtOAc/hexanes), **35** (459 mg, 72%) as a white solid: mp 131.5-133.0 °C; IR ($CHCl_3$ cast) 1709, 1644, 1379, 1363 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$) δ 7.64-7.44 (m, 5H, Ar**H**), 5.63 (s, 1H, NC**H**N), 4.35 (b d, 1H, $J$ = 4.6 Hz, C(O)C**H**N), 3.07 (s, 3H, $NCH_3$), 2.82-2.73 (m, 2H, $CH_2I$), 1.81-1.75, 1.60-1.48, 1.43-1.30, 1.20-1.10 (4 x m, 4H, 2 x $CH_2$); $^{13}C$ NMR (90 MHz, $CDCl_3$) δ 170.9, 136.2, 131.8, 129.0, 127.5, 79.8, 60.4, 40.9, 31.8, 26.6, 26.7, 4.0; MS (EI) 371.0244 (371.0256 calcd for $C_{14}H_{16}IN_2O_2$, $M^+$ - $(CH_3)_3C$ ); MS (CI, $NH_3$) 429 ($MH^+$). Anal. Calcd for $C_{18}H_{25}IN_2O_2$: C, 50.48; H, 5.88; N, 6.54. Found: C, 50.68; H, 5.91; N, 6.53.

**(2*R*, 5*R*, 3'*S*, 6'*R*)-5-[3-(3,6-Dihydro-2,5-dimethoxy-3-isopropyl-pyrazin-6-yl)-propyl]-1-benzoyl-2-*tert*-butyl-3-methyl-4-imidazolidinone (37).**

A solution of (2*S*)-(+)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine (95 mg, 0.52 mmol) in THF (2 mL) under argon at -78 °C was treated with BuLi (342 μL of a 1.55 M solution in hexane, 0.530 mmol). After stirring of the mixture for 10 min, **35** (217 mg, 0.51 mmol) in THF (2 mL) was added via cannula, and stirring was continued for 10 h. The solution was then gradually warmed to 0 °C over 6 h and quenched with water (200 μL). The THF was removed *in vacuo*, and the residue was partitioned into ether/water (20 mL/10 mL). The aqueous layer was extracted with ether (2 x 5 mL). The combined organic layers were washed with brine (5 mL), dried over $NaSO_4$, and concentrated *in vacuo*. Purification by flash chromatography (4 x 13 cm, 0-20% $CH_3CN/CH_2Cl_2$) gave

**37** (118 mg, 48%) as a solid: mp 116-118 °C; IR ($CH_2Cl_2$ cast) 2962, 1709, 1694, 1649, 1380 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$) δ 7.56-7.38 (m, 5H, Ar**H**), 5.61 (b s, 1H, NC**H**N), 4.30 (b s, 1H, NC**H**C(O)), 3.89-3.86 (dd, 1H, *J* = 3.8, 3.4 Hz, NC**H**CH$(CH_3)_2$), 3.78 (dt, 1H, *J* = 7.0, 3.5 Hz, NC**H**C=N), 3.66 (s, 6H, 2 x OC**H**$_3$), 3.04 (s, 3H, NC**H**$_3$), 2.21 (d x pentet, 1H, *J* = 7.0, 3.4 Hz, $(CH_3)_2$C**H**), 1.78-1.69, 1.60-1.49, 1.13-0.98 (3 x m, 4H, 2 x C**H**$_2$), 1.03 (s, 9H, $(CH_3)_3$C), 1.01 (d, 3H, *J* = 7.2 Hz, C**H**$_3$CH), 0.95-0.78 (m, 2H, C**H**$_2$), 0.73 (d, 3H, *J* = 8.2 Hz, CHC**H**$_3$); $^{13}C$ NMR (90 MHz, $CDCl_3$) δ 171.4, 163.6, 163.5, 136.8, 131.5, 128.8, 127.4, 80.0, 61.4, 60.7, 55.1, 52.4, 52.3, 41.0, 33.9, 31.9, 31.7, 30.4, 26.3, 19.0, 17.7, 16.6; exact mass 484.3046 (484.3050 calcd for $C_{27}H_{40}N_4O_4$). Anal. Calcd for $C_{27}H_{40}N_4O_4$: C, 66.92; H, 8.32; N, 11.56. Found: C, 66.97; H, 8.33; N, 11.47.

**Preparation of 37 by alkylation with 41**

The procedure used to prepare **35** was adapted. Thus the lithium enolate of (2*R*)-1-benzoyl-2-tert-butyl-3-methyl-4-imidazolidinone (78.9 mg, 0.300 mmol) was reacted with **41** (74.7 mg, 0.245 mmol) in THF (2.5 mL) and DMPU (0.4 mL) for 1.5 h at -78 °C. After work-up and purification (flash chromatography: 2 x 13 cm, 20-40% EtOAc/hexanes) as before, **37** (52.1 mg, 44%) was obtained as a white solid: mp 116-118 °C, spectral characteristics were identical with those of **37** determined above from **35**.

**(2R, 5R, 4'R)-5-(4'-Amino-4'-carbomethoxybutyl)-1-benzoyl-2-isopropyl-3-methyl-4-imidazolidinone (38).**

A solution of **37** (50.9 mg, 0.105 mmol) in 0.25 N HCl (3.5 mL) and acetone (1.1 mL) was stirred for 1.5 hr at room temperature. The acetone was removed *in vacuo*, and the aqueous residue was adjusted to pH 8 with solid $NaHCO_3$ and extracted with EtOAc (5 x 5 mL). The combined organic extracts were washed with brine (10 mL) and dried over $Na_2SO_4$. Concentration *in vacuo* and purification by flash chromatography (2 x 12 cm,

0-25% $CH_3CN$/EtOAc) gave **38** (27.2 mg, 67%) as a colorless oil. Characterization gave spectral characteristics in accord with those previously obtained on the analogous compound by Dr Y.-H. Song[142]: IR ($CH_2Cl_2$ cast) 3350, 2963, 1737, 1707, 1645 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 7.68-7.40 (m, 5H, Ar**H**), 5.64 (s, 1H, NC**H**N), 4.35 (s, 1H, NC**H**CO), 3.70 (s, 3H, OC**H**$_3$), 3.27 (dt, 1H, *J* = 4.8, 3.0 Hz C**H**$NH_2CO$), 3.08 (s, 3H, C**H**$_3N$), 1.86-0.76 (b m, 6H, C**H**$_2$C**H**$_2$C**H**$_2$), 1.05, (s, 9H, (C**H**$_3)_3C$); MS (CI, $NH_3$) 390 ($MH^+$). Anal. Calcd for $C_{21}H_{31}N_3O_4$: C, 64.76; H, 8.02; N, 10.79. Found: C, 64.58; H, 7.90; N, 10.64.

**(2*R*, 6*R*)-2,6-Diaminopimelic Acid (D,D-DAP, 39).**

Compound **37** (49.2 mg, 0.102 mmol) in 6 N HCl (5 mL) was heated to reflux for 27 h. The solution was cooled, extracted with $CH_2Cl_2$ (5 mL), and the aqueous layer was concentrated *in vacuo*. The residue was purified on a cellulose column (2.5 x 30 cm, 4:2:1 n-BuOH:$H_2O$:AcOH). The product **39** was converted to the dihydrochloride salt (15.0 mg, 56%). Characterization gave spectral values in accord with those previously obtained on the analogous compound by Dr. Y.-H. Song[142]: IR (MeOH cast) 3500-2400, 1735, 1660, 1585, 1490 $cm^{-1}$; $^1H$ NMR (200 MHz, $D_2O$) δ 4.08 (t, 2H, *J* = 6 Hz, 2x OCC**H**N), 2.20-1.90 (m, 4H, C**H**$_2$C**H**$_2$C**H**$_2$), 1.84-1.48 (m, 2H, CH$_2$C**H**$_2$CH$_2$); $^{13}C$ NMR (50 MHz, $D_2O$) δ 172.2, 52.8, 29.4, 20.4; MS (posFAB) 191.22 (191.10 calcd for $C_7H_{15}N_2O_4$, $MH^+$).

**Stereochemical Analysis of 39 and 2 by Marfey Derivatization.**

A sample of D,D-DAP (**39**) prepared above, as well as samples of D,D- and *meso*-DAP (**2**), prepared analogously by Dr Song as described earlier, were derivatized with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) according to the standard procedure.[120] The crude reaction mixtures were filtered (0.45 μm) and analyzed by HPLC directly on a Chemcosorb 5-ODS-UH reverse phase column (Chemcopak, 0.6 x 150 mm)

on a Beckman System Gold HPLC (see General Experimental section) eluting with 12 mM ammonium phosphate (pH 6.5) containing 4% DMF[145] with a $CH_3CN$ gradient of 0.5%/min for 30 min then 2%/min for 15 min at a flow rate of 1 ml/min with measurement at 340 nm. Retention times for the derivatives were: *meso*-(D,L)-DAP = 37.2 min, L,L-DAP = 38.3 min, D,D-DAP = 39.0 min. Detector reponse ratios for the different diastereomers were determined by derivatizing and analyzing the commercial mixture of isomers, assuming the statistical ratio of 1:1:2 for the D,D; L,L and D,L isomers respectively. The observed ratios were: DL/LL = 0.97, DL/DD = 0.88, DD/LL = 1.10.

Analysis results:

| DAP | % D,L | % L,L | % D,D | % d.e. |
|---|---|---|---|---|
| D,D-DAP, **39** | 12.7 | 0 | 87.3 | 74.6 |
| **39*** | 25.7 | 1.1 | 73.2 | 46.4 |
| *meso*-DAP, **2*** | 95.4 | 1.5 | 3.1 | 90.8 |

* Compounds were prepared by Dr. Y.-H. Song

**Preparation of 39 by Hydrolysis of 44**

The general literature method[133] was followed. Thus, a solution of **44** (10.0 mg, 18.1 μmol) and trifluoroacetic acid (90.0 μL, 1.17 mmol) in $CH_2Cl_2$ (300 μL) under argon was stirred at room temperature for 24 h. The solvent was removed *in vacuo* and the residue was dissolved in 0.75 N HCl (2 mL) and stirred in a sealed flask with cation exchange resin (BioRad AG50W-X8, $H^+$ form, 4 mL) at 100 °C for 5 days. The mixture was then cooled to room temperature and the solution was filtered and concentrated to dryness *in vacuo*. The residue was dissolved in water (1 mL) and reapplied to the cation exchange resin in a column. The resin was washed with ethanol (6 mL), and water (6 mL), and eluted with 10% aqueous ammonia solution. The ninhydrin-positive fractions were

combined and concentrated to dryness *in vacuo*. The residue was dissolved in water and freeze-dried to give **39** (2.90 mg, 84%) as an amorphous white solid. Spectral values were consistent with those for **39** from **37**. Derivatization with Marfey's reagent and analysis by HPLC was performed as described in the preceding experimental procedure. The observed diastereomeric ratios were: D,D-DAP : *meso*-DAP : L,L-DAP, 83 : 15 : 2.

**Alternative procedure:** A sample of **44** (8.55 mg, 15.5 μmol) was hydrolyzed in 6N HCl (1 mL) at 100 °C for 45 h in a sealed flask. The solution was cooled to room temperature and extracted with $CHCl_3$ (2 x 1 mL). The aqueous phase was concentrated to dryness *in vacuo*. The residue was dissolved in water and freeze-dried to give **39** (5.06 mg, quantitative) as a foam. Derivatization with Marfey's reagent and analysis by HPLC as described for **39** from **37** led to the following diastereomeric ratios being observed: D,D-DAP : *meso*-DAP, 97 : 3.

**(3*S*, 6*R*)-6-(3'-Bromopropyl)-3,6-dihydro-2,5-dimethoxy-3-isopropyl-pyrazine (41)**

The procedure of Schollkopf *et al* [152] for similar compounds was adapted. A stirred solution of (3*S*)-(+)-3,6-dihydro-2,5-dimethoxy-3-isopropylpyrazine (179 μL, 1.00 mmol) in dry THF (3 mL) under argon was treated with BuLi (0.677 mL of a 1.55 M solution in hexane, 1.05 mmol) at -78 °C, and then stirred for 15 min. DMPU (244 μL, 2.00 mmol) and 1,3-dibromopropane (305 μL, 3.00 mmol, filtered through basic alumina) were then added, and the reaction mixture was stirred at -78 °C for 2 h before warming to room temperature. The THF was removed *in vacuo*, and the residue was partitioned between ether and water (10 mL each). The aqueous layer was extracted with ether (3 x 5 mL). The combined organic layers were washed with brine (5 mL), dried over $NaSO_4$, and concentrated *in vacuo* to a yellow oil. Purification by flash chromatography (4 x 12 cm, 0-5% EtOAc/hexanes) gave **41** (98.0 mg, 32%) as a colorless oil: IR ($CH_2Cl_2$ cast) 2960, 2872, 1693, 1657, 1436, 1239 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 4.07-3.99 (m,

1H, C**H**$(CH_2)_3$Br), 3.96 (d, 1H, $J$ = 3 Hz, C**H**CH$(CH_3)_2$), 3.70 (s, 3H, OC**H**$_3$), 3.68 (s, 3H, OC**H**$_3$), 3.44 (t, 2H, $J$ = 6 Hz, C**H**$_2$Br), 2.40-2.20 (d x sept, 1H, $J$ = 7, 3 Hz, $CH_3$C**H**$CH_3$), 2.05-1.75 (m, 4H, C**H**$_2$C**H**$_2$C**H**$_2$Br), 1.04 and 0.70 (2 x d, 6H, $J$ = 7 Hz, C**H**$_3$CHC**H**$_3$); exact mass 306.0763 (306.0766 calcd for $C_{12}H_{21}{}^{81}BrN_2O_2$), 304.0780 (304.0786 calcd for $C_{12}H_{21}{}^{79}BrN_2O_2$).

**(2*S*)-2-*tert*-Butyl-3-methyl-4-imidazolidinone, (*S*)-Mandelate Salt (42)**

The literature procedure[133,146] was followed. The mother liquor remaining after fractional crystallization of the (2*R*)-2-*tert*-butyl-3-methyl-4-imidazolidinone (*R*)-mandelate salt from the diastereomeric mixture of (*R*)-mandelate salts was obtained from a colleague, Dr. Yunlong Pu. The solution, containing the (2*S*)-amine (*R*)-mandelate salt (59g, 191 mmol) and (2*R*)-amine (*R*)-mandelate salt (17 g, 55 mmol) in acetone, was concentrated *in vacuo* to a yellow oil, then redissolved in $CH_2Cl_2$ (300 mL), and extracted with 2 N NaOH (130 mL, 0.26 mol). The aqueous layer was acidified with 50% aqueous $H_2SO_4$, cooled to 4 °C, and the (*R*)-(-)-mandelic acid recovered by filtration as the sodium salt. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to a yellow oil. The oil was dissolved in hot acetone (70 mL) containing (*S*)-(+)-mandelic acid (40.0 g, 0.260 mol) and the solution was allowed to cool slowly to room temperature. It was found to be necessary to cool the solution to -20 °C to induce crystallization. Filtration and drying *in vacuo* gave a pale yellow solid (40.3 g) which was recrystallized from acetone (70 mL) again, affording **42** (18.9 g, 32% ) as pale yellow crystals: mp 113.5-115.0 °C (lit.[133] mp 115.5-116.5 °C); $[\alpha]_D$ = +87.25° (c = 1.05 in EtOH, 98% e.e.) (lit.[133] $[\alpha]_D$ = +89° (c = 1.1 in EtOH)); IR ($CH_2Cl_2$/MeOH cast) 3219 (br), 3045, 2958, 1707, 1647 cm$^{-1}$; $^1$H NMR (300 MHz, $CD_3OD$) δ 7.48-7.27 (m, 5H, Ar**H**), 5.11 (s, 1H, C**H**OH), 4.23 (s, 1H, C**H**C$(CH_3)_3$), 3.48 (d, 1H, $J$ = 16 Hz, C**H**$_a$HC(O)), 3.40 (d, 1H, $J$ = 16 Hz, CH**H**$_b$C(O)), 2.93 (s, 3H, $NCH_3$), 0.98 (s, 9H, C$(CH_3)_3$); $^{13}$C NMR (75.5 MHz,

$CD_3OD$) δ 176.5, 176.3, 141.1, 129.4, 129.1, 127.9, 86.1, 74.3, 49.5, 38.2, 31.6, 25.8. Anal. Calcd. for $C_{16}H_{24}N_2O_4$: C, 62.32; H, 7.84; N, 9.08. Found: C, 62.37; H, 7.79; N, 9.04.

**(2*R*)-2-*tert*-Butyl-1-*tert*-butyloxycarbonyl-3-methyl-4-imidazolidinone (43)** The literature preparation of **43** was followed.[133] A suspension of **42** (3.08 g, 10.0 mmol) in $CH_2Cl_2$ (35 mL) was washed with 2 N NaOH (7.5 mL, 15 mmol). The aqueous layer was extracted with $CH_2Cl_2$ (10 mL), the combined organic layers were washed with brine (20 mL), and the solvent was removed *in vacuo*. The residual oil was dissolved with cooling in an acetone solution (20 mL) of di-*tert*-butylpyrocarbonate (2.80 g, 12.7 mmol) and *N*,*N*-4-dimethylaminopyridine (0.120 g, 1.00 mmol). The mixture was stirred at room temperature for 19 h, and triethylamine (1.39 mL, 10.0 mmol) was added. This was followed 2 h later by the addition of water (1 mL) and stirring for another 2 h. The acetone was removed *in vacuo*. The residue was dissolved in ether (30 mL), washed with 1 N HCl (2 x 15 mL), saturated $NaHCO_3$ (15 mL), and dried over $MgSO_4$. Concentration *in vacuo* gave **43** as a colorless oil which solidified on standing (2.07 g, 81%): mp 65-68 °C (lit.[133] 68-70 °C); $[\alpha]_D$ = +13.45° (c = 1.14 in $CH_2Cl_2$, 92% e.e.) (lit.[133] $[\alpha]_D$ = -14.6° (c = 1.18 in $CH_2Cl_2$) for the opposite enantiomer); IR ($CHCl_3$ cast) 3045, 2943, 1621, 1437, 1348, 1126, 1104 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 4.86 (b s, 1H, NC**H**N), 4.05 (b d, 1H, *J* = 16.0 Hz, C(O)C**H**$_a$HN), 3.65 (d, 1H, *J* = 16.0 Hz, C(O)CH**H**$_b$N), 2.92 (s, 3H, NC**H**$_3$), 1.39 (s, 9H, (C**H**$_3$)$_3$CO), 0.90 (s, 9H, (C**H**$_3$)$_3$CCH); $^{13}C$ NMR (50 MHz, $CDCl_3$) δ 170.3 (**C**(O)NCH$_3$), 154.3 (**C**O$_2$C(CH$_3$)$_3$), 82.3 (NCHN), 81.0 ((CH$_3$)$_3$**C**O), 49.6 (**C**H$_2$), 39.4 ((CH$_3$)$_3$**C**CH), 31.3 (NCH$_3$), 28.1 ((**C**H$_3$)$_3$CO), 25.6 ((**C**H$_3$)$_3$CCH); exact mass 257.1855 (257.1865 calcd for $C_{13}H_{25}N_2O_3$). Anal. Calcd. for $C_{13}H_{25}N_2O_3$: C, 60.67; H, 9.79; N, 10.89. Found: C, 60.69; H, 9.45; N, 10.86.

**Bis-1,3-[5-((2*R*,5*R*)-2-*tert*-butyl-1-*tert*-butyloxycarbonyl-3-methyl-4-imidazolidinone)]propane (44)**

The general alkylation procedure of Fitzi and Seebach[133] was followed. A solution of **43** (141 mg, 0.550 mmol) in THF (1 mL) was added via cannula to a solution of LDA (prepared by addition of BuLi (0.4 mL of a 1.5 M solution in hexanes, 0.60 mmol) to *N,N*-diisopropylamine (84.0 μL, 0.600 mL) in THF (0.2 mL)) at -78 °C under argon, and the pale yellow solution was stirred for 40 min. 1,3-Dibromopropane (24.5 μL, 0.250 mmol, filtered through activated basic alumina) was then added, followed by DMPU (0.32 mL). The solution was slowly warmed to -5 °C over 7 h, stirred for 10 h, then allowed to warm to room temperature. Saturated $NH_4Cl$ (0.3 mL) was added and the volatile solvents were removed *in vacuo*. The residue was redissolved in ether (10 mL), washed with 2 N citric acid (2 x 5 mL), saturated $NaHCO_3$ (5 mL), brine (5 mL), and dried over $Na_2SO_4$. Concentration *in vacuo* gave a white solid (151 mg) which was dissolved in hot diisopropyl ether (10 mL). Cooling and filtration gave a white solid which was dried *in vacuo* to afford **44** (77.7 mg, 56%): IR ($CHCl_3$ cast) 2973, 2362, 1704, 1383, 1366 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$) δ 4.95 (s, 2H, 2 x NC**H**N), 4.04-3.95 (m, 2H, 2 x C(O)C**H**N), 2.95 (s, 6H, 2 x NC**H**$_3$), 2.51-2.22 (b m, 2H, C**H**$_a$HCH$_2$C**H**$_a$H), 1.96-1.77 (m, 2H, CH**H**$_b$CH$_2$CH**H**$_b$), 1.48 (s, 18H, 2 x (C**H**$_3$)$_3$CO), 0.96 (s, 18H, 2 x (C**H**$_3$)$_3$CCH), 0.82-0.61 (m, 2H, CH$_2$C**H**$_2$CH$_2$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 171.6 (**C**(O)NCH$_3$), 153.2 (**C**O$_2$C(CH$_3$)$_3$), 80.9 ((CH$_3$)$_3$**C**O), 80.5 (N**C**HN), 59.2 (NCHC(O)N), 40.8 ((CH$_3$)$_3$**C**CH), 31.6 (NCH$_3$), 29.6 (**C**H$_2$), 28.2 ((**C**H$_3$)$_3$CO), 26.4 ((**C**H$_3$)$_3$CCH), 16.6 (**C**H$_2$); exact mass 495.3184 (495.3183 calcd for $C_{25}H_{43}N_4O_6$, $M^+$-$C_4H_9$), MS (CI, $NH_3$) 553 (100%, $MH^+$). Anal. Calcd. for $C_{29}H_{52}N_4O_6 \cdot 1/2\ H_2O$: C, 62.00; H, 9.51; N, 9.97. Found: C, 61.75; H, 9.46; N, 9.84.

**_N_-Succinyl-L-allylglycine, Dimethyl Ester [§] (45)**

A solution of L-(-)-allylglycine (2.0 g, 17.4 mmol) in aqueous $KHCO_3$ (50 mL, 4.36 g, 42.7 mmol) was stirred at room temperature. Succinic anhydride (2.61 g, 26.1 mmol) was added in portions over 20 min. The solution was stirred for 1 h, acidified with concentrated aqueous HCl to pH 2.0, and extracted into EtOAc (4 x 50 mL). The combined organic extracts were dried over $Na_2SO_4$ and concentrated *in vacuo* to a white solid. This was treated with an excess of an ethereal solution of $CH_2N_2$, and stirred at room temperature for 10 min. The excess $CH_2N_2$ was then destroyed with glacial acetic acid. Removal of the solvent *in vacuo* followed by drying under high vacuum (0.05 mm Hg) for 12 h afforded **45** (3.37g, 80%) as a viscous oil: $[\alpha]_D$ = +36.24° (c = 43.5, $CHCl_3$); IR ($CHCl_3$ cast) 3313, 3003, 2982, 2954, 1741, 1678, 1657, 1536 $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3$) δ 6.50 (d, 1H, *J* = 7.5 Hz, N**H**), 5.75-5.64 (m, 1H, $CH_2$=C**H**), 5.15-5.10 (m, 2H, C$H_2$=CH), 4.66 (dt, 1H, *J* = 7.8, 6.3 Hz, $CH_2$C**H**NH), 3.74 (s, 3H, OC$H_3$), 3.69 (s, 3H, OC$H_3$), 2.73-2.64 (m, 2H, C$H_2$CHNH), 2.59-2.44 (m, 4H, C$H_2$C$H_2$); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) δ 172.94 (**C**$O_2$), 171.92 (**C**$O_2$), 170.9 (**C**ONH), 132.1 ($CH_2$=**C**H), 118.7 (**C**$H_2$=CH), 52.0 (**C**HNH), 51.5 (2 x OC$H_3$), 36.1 (**C**$H_2$CHNH), 30.4 (**C**$H_2$C$H_2$), 28.9 ($CH_2$**C**$H_2$); exact mass 243.1104 (243.1107 calcd for $C_{11}H_{17}NO_5$). Anal. Calcd for $C_{11}H_{17}NO_5$: C, 54.31; H, 7.04; N, 5.76. Found: C, 54.17; H, 6.99; N, 5.78.

**Methyl Glyoxylate (46)**

The method of Kelly *et al* was followed.[156] Periodic acid (45.6 g, 0.200 mol) was added in portions over 1 h to a stirred solution of dimethyl DL-tartrate (35.6 g, 0.200 mol) in ether (400 mL) under argon with cooling (10 °C). The cloudy suspension was stirred for 45 min until a clear solution and a white precipitate formed. The mixture was filtered and the filtrate was dried overnight over molecular sieves (4Å, 30 g). Concentration *in vacuo* and distillation from phosphorus pentoxide under vacuum (1.5 mm Hg) into a cooled receiving

flask (dry ice/acetone) afforded **46** (13.5 g, 38%) as a viscous colorless oil: bp 30 °C at 1.5 mm Hg (lit.[202] bp 47-54 °C at 25 mm Hg, lit.[156] bp 45-50 °C at 29 mm Hg); IR ($CH_2Cl_2$ cast) 2960, 1756 (br), 1441, 1232, 1139 (br), 978 (br) $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) δ 9.41 (s, 1H **CHO**), 3.94 (s, 3H, $OCH_3$); exact mass 88.0165 (88.0160 calcd for $C_3H_4O_3$).

**(6*S*)-*E*-6-Amino-2-hydroxy-*N*-succinylhept-4-en-1,7-dioic acid, Trimethyl Ester (47)**

The general procedure of Agouridas *et al* [155] was adapted. All transfers were done under argon. Distilled methyl glyoxylate (**46**) (1.85 g, 21.0 mmol) in dry $CH_2Cl_2$ (15 mL) was added via cannula to a stirred suspension of anhydrous $FeCl_3$ (6.5 g, 40.0 mmol) under argon at room temperature. After 30 min a solution of **45** (2.43 g, 10.0 mmol) in $CH_2Cl_2$ (15 mL) was added dropwise via cannula over 20 min with cooling in ice. The suspension was warmed to room temperature and allowed to stir for a further 20 h. The resulting black mixture was poured into water (200 mL) and extracted with $CHCl_3$ (1 x 100 mL, 2 x 50 mL). The combined organic extracts were washed with 1 N HCl (100 mL) and brine (75 mL) and dried over $MgSO_4$. Concentration *in vacuo* gave a pale yellow oil which was purified by flash chromatography (4 x 15 cm, 75-85% EtOAc/hexanes) to afford **47** (2.18 g, 65%) as a colorless oil: IR ($CHCl_3$ cast) 3140, 2950, 1738, 1656, 1531, 1437 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$) δ 6.22 (br s, 1H, **NH**), 5.82-5.72 (m, 1H, **CH**=CHCHN), 5.62 (dd, 1H, $J$ = 6.0, 15.5 Hz, CH=**CH**CHN), 5.05 (dd, 1H, $J$ = 12.0, 6.0 Hz, **CH**NH), 4.25-4.22 (dd, 1H, $J$ = 6.6, 4.5 Hz, **CH**OH), 3.74 (s, 3H, $OCH_3$), 3.73 (s, 3H, $OCH_3$), 3.68 (s, 3H, $OCH_3$), 2.76-2.38 (br m, 6H, 3 x $CH_2$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 174.2, 173.2, 171.3, 171.0, 128.3, 127.4, 69.8, 53.8, 52.4, 52.1, 51.6, 36.8, 30.3, 28.9; exact mass 331.1269 (331.1267 calcd for $C_{14}H_{21}NO_8$).

**(6*S*)-6-Amino-2-hydroxy-*N*-succinylheptan-1,7-dioic acid, Trimethyl Ester (48)**

A solution of **47** (2.00 g, 6.04 mmol) in MeOH (10 mL) was added to a suspension of 5% Pd on carbon (0.206 g, presaturated with hydrogen) in MeOH (50 mL) and stirred under an atmosphere of hydrogen for 45 h. The catalyst was removed by filtration through Celite and the solution was concentrated *in vacuo* to a pale yellow oil. Purification by flash chromatography (5 x 14 cm, 70-80% EtOAc/hexanes) afforded **48** (1.84 g, 91%) as a colorless oil: IR ($CHCl_3$ cast) 3140, 2950, 1738, 1656, 1531, 1437 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$) δ 6.24 (t, 1H, $J$ = 7.0 Hz, N**H**), 4.63-4.58 (m, 1H, C**H**NH), 4.20-4.15 (m, 1H, C**H**OH), 3.78 (s, 3H, OC**H**$_3$), 3.75 (s, 3H, OC**H**$_3$), 3.69 (s, 3H, OC**H**$_3$), 2.90 (br s, 1H, O**H**), 2.76-2.55 (b m, 4H, succinyl C**H**$_2$C**H**$_2$), 1.89-1.66 (b m, 4H, C**H**$_2$CH$_2$C**H**$_2$), 1.50-1.20 (m, 2H, CH$_2$C**H**$_2$CH$_2$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 175.1, 173.2, 172.8, 171.3, 70.0, 52.4, 52.2, 51.8, 51.7, 33.4, 31.7, 30.5, 29.0, 20.5; exact mass 333.1414 (333.1424 calcd for $C_{14}H_{23}NO_8$).

**(6*S*)-6-Amino-2-oxo-*N*-succinylheptan-1,7-dioic acid, Trimethyl Ester (49)**

The general procedure of Dess and Martin[159] was modified. The α-hydroxy ester **48** (0.621 g, 1.87 mmol) in dry $CH_2Cl_2$ (5 mL) was added via cannula to a stirred suspension of the Dess-Martin periodinane[203] (1.29 g, 3.00 mmol) in $CH_2Cl_2$ (10 mL) under argon at room temperature. After stirring for 1.5 h, the mixture was diluted with EtOAc (25 mL) and poured into saturated $NaHCO_3$ (25 mL) containing $Na_2S_2O_3$ (4.43 g, 28.0 mmol). The aqueous layer was extracted with EtOAc (15 mL). The combined organic layers were washed with saturated $NaHCO_3$ (15 mL) and brine (15 mL), and dried over $NaSO_4$. Concentration *in vacuo* gave a cloudy yellow oil which was purified by flash chromatography (2.5 x 17.5 cm, 89:10:1 $CH_2Cl_2$:$CH_3CN$:EtOH) to afford **49** (0.302 g, 49%) as an oil: $[\alpha]_D$ = +18.4° (c = 4.762 in $CHCl_3$); IR ($CH_2Cl_2$ cast) 3350, 2956, 1734,

1678, 1660 $cm^{-1}$; $^1H$ NMR (360 MHz, $CDCl_3$) δ 6.26 (d, 1H, *J* = 7.6 Hz, **NH**), 4.61-4.55 (m, 1H, **CH**NH), 3.83 (s, 3H, $OCH_3$), 3.71 (s, 3H, $OCH_3$), 3.65 (s, 3H, $OCH_3$), 2.85 (t, 2H, *J* = 6.9 Hz, $CH_2C(O)CO_2CH_3$), 2.67-2.59 (m, 2H, succinyl $CH_2$), 2.54-2.47 (m, 2H, succinyl $CH_2$), 1.92-1.82 (m, 1H, $CH_2CH_aHCHNH$), 1.76-1.62 (m, 3H, $CH_2CHH_bCHNH$); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) δ 193.4 ($C(O)CO_2CH_3$), 173.2 ($CO_2CH_3$), 172.5 ($CO_2CH_3$), 171.2 (C(O)NH), 161.2 ($C(O)CO_2CH_3$), 52.7 (CHNH), 52.1 ($OCH_3$), 51.5 (2 x $OCH_3$), 38.3 ($CH_2$), 31.4 ($CH_2$), 30.4 ($CH_2$), 28.9 ($CH_2$), 18.3 ($CH_2$); exact mass 331.1262 (331.1267 calcd for $C_{14}H_{21}NO_8$). Anal. Calcd for $C_{14}H_{21}NO_8$: C, 50.75; H, 6.39; N, 4.23. Found: C, 50.34; H, 6.46; N, 4.47.

**(6*S*)-6-Amino-2-oxo-*N*-succinylheptan-1,7-dioic Acid, Tri-Lithium Salt (50)**

Lithium hydroxide monohydrate (47.5 mg, 1.13 mmol) was added to a vigorously stirred solution of **49** (125 mg, 0.377 mmol) at 0 °C, thereby generating a strong yellow color. The mixture was stirred for 1 h, the THF was removed *in vacuo*, and the residue was freeze dried. The residue was precipitated from ethanol/acetone according to Gilvarg's method[154] for the isolation of this compound; the residue was dissolved in water (1 mL) and added dropwise to a rapidly stirred mixture of ethanol/acetone (1:2, 20 mL). A white precipitate rapidly formed with a cloudy white supernatant. The precipitate was filtered, redissolved in water (0.5 mL), and reprecipitated by addition to ethanol/acetone (15 mL) again. The recovered precipitate was dissolved in water and freeze dried to give **50**[154] (119 mg, polyhydrate; theor. yield = 116 mg) as a foam. The combined filtrates from each precipitation step were concentrated *in vacuo* and then freeze dried to give a foam (68.2 mg).

For **50**: $[\alpha]_D$ = +6.4° (c = 0.683 in $H_2O$); IR (KBr disk) 3482, 2970, 1675, 1671, 1596, 1420 $cm^{-1}$; $^1H$ NMR (360 MHz, $D_2O$ + $CH_3CN$) δ 3.90-3.76 (m, 1H, **CH**NH), 2.48-2.40 (m, <2H, $CH_2C(O)CO_2Li$, enolizable, exchanges with $^2H$), 2.28-2.07 (m, 4H,

succinyl $CH_2CH_2$), 1.56-0.97 (b m, 4H, $CH_2CH_2CHNH$); MS (posFAB, Cleland) 309 ($M(D_2)H^+$), 307 ($M^+$).

**Ethyl 8-*E*-6-Carboethoxynon-8-enoate ¶ (51)**

The literature preparation[173] of this compound was modified. Ethyl allyl-2-cyclohexanone-carboxylate[172] (37.0 g, 176 mmol) in dry EtOH (100 mL) was added dropwise to a stirred solution of NaOEt [prepared from Na metal (4.05 g, 176 mmol)] in EtOH (100 mL). The mixture was heated to reflux for 2 h, and then the EtOH was removed *in vacuo* and water (100mL) was added. The mixture was extracted with ether (4 x 300 mL). The combined organic layers were washed with brine (150 mL), dried over $MgSO_4$, and concentrated *in vacuo* to a light yellow oil. Distillation *in vacuo* (12 mm Hg) gave **51** (38.4 g, 85%) as a colorless oil: bp 161-163 °C at 12 mm Hg (lit.[173] bp 161 °C at 15 mm Hg); $^1H$ NMR (300 MHz, $CDCl_3$) δ 5.80-5.67 (m, 1H, $CH_2$=C**H**), 5.08-4.98 (m, 2H, $CH_2$=CH), 4.13 (q, 2H, $J$ = 7.1 Hz, $CO_2CH_2CH_3$), 4.12 (q, 2H, $J$ = 7.1 Hz, $CO_2CH_2CH_3$), 2.47-2.16 (b m, 3H, $CH_2$=CHC$H_2$CH), 2.29 (t, 2H, $J$ = 7.5 Hz, $CH_2CO_2$), 1.70-1.43 (b m, 4H, 2 x $CH_2$), 1.33 (q, 2H, $J$ = 7.4 Hz, $CH_2$), 1.25 (t, 6H, $J$ = 7.1 Hz, 2 x $CO_2CH_2CH_3$); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) δ 175.2, 173.3, 135.3, 116.5, 60.0, 50.0, 36.3, 34.0, 31.2, 26.6, 24.7, 14.2, 14.1; exact mass 256.1676 (256.1675 calcd for $C_{14}H_{24}O_4$).

**1-Methyl, 11-Ethyl 4-*E*-6-Carboethoxy-2-hydroxyundec-4-en-1,11-dioate¶ (52)**

The general method of Agouridas *et al*[155] was followed. Methyl glyoxylate (**46**, 1.81 g, 20.5 mmol) in dry $CH_2Cl_2$ (20 mL) was added to a stirred suspension of anhydrous $FeCl_3$ (6.66 g, 41.1 mmol) in $CH_2Cl_2$ (20 mL) under argon. The mixture was stirred for 30 min and a solution of **51** (2.64 g, 10.3 mmol) in $CH_2Cl_2$ (20 mL) was added dropwise with cooling (ice-bath). The mixture was stirred at room temperature for 22 h and then poured into ice (50g) and extracted with $CHCl_3$ (3 x 150 mL). The combined organic extracts

were washed with 1 N HCl (100 mL), and brine (100 mL), and dried over $MgSO_4$. Concentration *in vacuo* gave a brown oil which was purified by flash chromatography (4 x 20 cm, 33% EtOAc/hexanes) to afford **52** (2.30 g, 65%) as a clear oil: IR 3460 (br), 2940, 1733, 1177 $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) δ 5.57-5.53 (m, 2H, C**H**=C**H**), 4.26 (dt, 1H, *J* = 6.0, 4.9 Hz, $CH_2CHOH$), 4.13 (q, 2H, *J* = 7.2 Hz, $OCH_2CH_3$), 4.12 (q, 2H, *J* = 7.2 Hz, $OCH_2CH_3$), 3.77 (s, 3H, $OCH_3$), 3.05 (d, 1H, *J* = 6.0 Hz, CHO**H**), 2.96 (dt, 1H, *J* = 7.0, 6.0 Hz, $CH=CHCHCO_2$), 2.57-2.51 (m, 1H, $CH_aHCH=CH$), 2.46-2.39 (m, 1H, $CHH_bCH=CH$), 2.29 (m, 2H, *J* = 7.5 Hz, $CH_2CH_2CO_2CH_2CH_3$), 1.78-1.46 (b m, 4H, 2 x $CH_2$), 1.38-1.23 (m, 2H, $CH_2$), 1.26 (t, 3H, *J* = 7.2 Hz, $OCH_2CH_3$), 1.25 (t, 3H, *J* = 7.2 Hz, $OCH_2CH_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 174.5, 173.9, 173.4, 131.8, 126.8, 70.1, 60.4, 60.1, 52.2, 48.8, 37.2, 33.9, 31.8, 26.3, 24.4, 14.0; exact mass 344.1834 (344.1835 calcd for $C_{17}H_{28}O_7$).

**1-Methyl, 11-Ethyl 4-*E*-6-Carboethoxy-2-hydroxyundecan-1,11-dioate (53)**

A solution of **52** (2.10 g, 6.10 mmol) in MeOH (50 mL) was treated with 5% Pd on carbon (200 mg), and the mixture was stirred under one atmosphere of $H_2$ at room temperature for 24 h. The mixture was filtered through Celite and concentrated *in vacuo* to a light yellow oil. Purification by flash chromatography (4 x 15 cm, 35% EtOAc/hexanes) gave **53** (1.87 g) as a colorless oil. $^1H$ and $^{13}C$ NMR indicated it was a mixture of the desired product and the corresponding 11-methyl ester (arising from transesterification with the solvent).

For **53**: IR ($CHCl_3$ cast) 3510, 2950, 1733, 1201 (br) $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) δ 4.20-4.11 (m, 1H, C**H**OH), 4.17-4.09 (m, 2.9H, $CHCOCH_2CH_3$, 0.45 x $CH_2COCH_2CH_3$), 3.78 (s, 3H, $CH(OH)CO_2CH_3$), 3.66 (s, 1.65H, 0.55 x $CH_2COCH_3$), 3.11 (d, 1H, *J* = 5.6 Hz, CHO**H**), 2.38-2.33 (m, 1H, $CHCO_2CH_2CH_3$), 2.30 (t, 1.10 H, *J* = 7.2 Hz, 0.55 x $CH_2CH_2CO_2CH_3$), 2.28 (t, 0.9H, *J* = 7.2 Hz,

0.45 x $CH_2CH_2CO_2CH_2CH_3$), 1.82-1.72 (m, 1H, $CH_aHCHOH$), 1.69-1.57 (m, 5H, $CHH_bCHOH$, 2 x $CH_2$), 1.51-1.38 (m, 4H, 2 x $CH_2$), 1.31 (q, 2H, $J$ = 7.5 Hz, $CH_2$), 1.25 (t, 4.35H, $J$ = 7.1 Hz, $CHCO_2CH_2CH_3$, 0.45 x $CH_2CO_2CH_2CH_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 175.7, 175.3, 173.7, 173.3, 70.0, 59.9, 59.8, 52.1, 51.2, 45.1, 34.0, 33.9, 33.6, 31.8, 26.6, 24.6, 22.5, 14.1, 14.0; exact mass 300.1569 (300.1573 calcd for $C_{15}H_{24}O_6$, $M^+$-$CH_3CH_2OH$); MS (CI, $NH_3$) 364 ($MNH_4^+$ for diethyl ester, 100%), 350 ($MNH_4^+$ for dimethyl ester, 99.9%).

**1-Methyl, 11-Ethyl 6-Carboethoxy-2-oxoundecan-1,11-dioate [§] (54a) and 1, 11-Dimethyl 6-Carboethoxy-2-oxoundecan-1,11-dioate [§] (54b)**

The procedure for **49** was followed. Thus the Dess-Martin periodinane (0.546 g, 1.29 mmol) in $CH_2Cl_2$ (15 mL) was added dropwise to a stirred solution of the mixture of esters **53** (0.223 g, 0.659 mmol ) under argon. Stirring was continued for 1.5 h then the mixture was worked up and purified by flash chromatography (30% EtOAc/hexanes) to give **54a** (32.8 mg, dimethyl ester) and **54b** (124 mg, diethyl ester) with a combined yield of 63% from **52**.

For **54a**: IR ($CHCl_3$ cast) 2942, 1732, 1259, 1183 $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) δ 4.13 (q, 2H, $J$ = 7.1 Hz, $CO_2CH_2CH_3$), 4.12 (q, 2H, $J$ = 7.2 Hz, $CO_2CH_2CH_3$), 3.86 (s, 3H, $CO_2CH_3$), 2.85 (t, 2H, $J$ = 5.5 Hz, $CH_2C(O)CO_2CH_3$), 2.38-2.30 (m, 1H, $CHCO_2CH_2CH_3$), 2.28 (t, 2H, $J$ = 7.5 Hz, $CH_2CO_2CH_2CH_3$), 1.67-1.58 (m, 6H, 3 x $CH_2$), 1.51-1.45 (m, 2H, $CH_2$), 1.31 (q, 2H, $J$ = 7.6 Hz, $CH_2$), 1.26 (t, 3H, $J$ = 7.2 Hz, $CO_2CH_2CH_3$), 1.25 (t, 3H, $J$ = 7.1 Hz, $CO_2CH_2CH_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 193.6, 175.6, 173.5, 161.4, 60.2, 60.1, 52.8, 45.2, 39.0, 34.1, 31.9, 31.4, 26.8, 24.8, 20.7, 14.3, 14.2.

For **54b**: $^1H$ NMR (400 MHz, $CDCl_3$) δ 4.14 (q, 2H, $J$ = 7.1 Hz, $CO_2CH_2CH_3$), 3.87 (s, 3H, $C(O)CO_2CH_3$), 3.66 (s, 3H, $CO_2CH_3$), 2.84 (t, 2H, $J$ = 7.1 Hz, $CH_2C(O)CO_2CH_3$), 2.38-2.32 (m, 1H, $CHCO_2CH_2CH_3$), 2.30 (t, 2H, $J$ = 7.5 Hz,

$CH_2CO_2CH_2CH_3$), 1.67-1.58 (m, 6H, 3 x $CH_2$), 1.51-1.45 (m, 2H, $CH_2$), 1.31 (q, 2H, $J$ = 7.6 Hz, $CH_2$), 1.26 (t, 3H, $J$ = 7.1 Hz, $CO_2CH_2CH_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ 193.6, 175.6, 173.9, 161.4, 60.2, 52.8, 51.4, 45.1, 39.0, 33.8, 31.9, 31.4, 26.7, 24.7, 20.7, 14.2.

**_N_-Succinyl-3-butenylamine Methyl Ester [1] (55)**

Succinic anhydride (5.58 g, 55.8 mmol) was added in portions over 20 min to a stirred solution of 3-butenylamine hydrochloride[204] (3.00 g, 27.9 mmol) in an aqueous $KHCO_3$ solution (50 mL, 6.98 g, 69.8 mmol) at 4 °C. The solution was maintained at pH 9.5 by addition of 10 N NaOH. After stirring for a further 40 min, the solution was adjusted to pH 1.0 with concentrated aqueous HCl and extracted with EtOAc (4 x 150 mL). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo* to a white solid containing a mixture of the desired succinamide and succinic acid. The mixture was dissolved in EtOAc (50 mL) and treated dropwise with an ethereal solution of diazomethane until the yellow color persisted. Excess diazomethane was destroyed by the addition of a few drops of glacial acetic acid. Concentration *in vacuo* gave a light yellow oil which was dried *in vacuo* for 3 days to remove dimethyl succinate. The residue was then purified by flash chromatography (4 x 23 cm, 50% EtOAc/hexanes) to afford **55** (2.21 g, 43%) as an oil: IR ($CHCl_3$ cast) 3305, 1740, 1649, 1437, 1168 $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3$) δ 6.15 (b s, 1H, N**H**), 5.84-5.70 (m, 1H, $CH_2$=C**H**), 5.13-5.05 (m, 2H, $CH_2$=CH), 3.69 (s, 3H, $OCH_3$), 3.32 (dt, 2H, $J$ = 6.8, 6.7 Hz, $CH_2NHC(O)$), 2.67 (t, 2H, $J$ = 6.8 Hz, $CH_2CH_2$), 2.48 (t, 2H, $J$ = 6.8 Hz, $CH_2CH_2$), 2.26 (dt, 2H, $J$ = 6.8, 6.4 Hz, CH2=CHC**H**$_2$); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) δ 173.3, 171.2, 135.1, 116.8, 51.6, 38.4, 33.5, 30.8, 29.2; exact mass 185.1054 (185.1052 calcd for $C_9H_{15}NO_3$). Anal. Calcd for $C_9H_{15}NO_3$: C, 58.36; H, 8.16; N, 7.56. Found: C, 58.20; H, 8.34; N, 7.64.

**Methyl *N*-(4'-methylsuccinyl)-6-amino-2-hydroxy-4-hexenoate ¶ (56)**

The preparation followed the same method as used for **51**. Thus reaction of **55** (92.5 mg, 0.500 mmol) with methyl glyoxylate (**46**, 176 mg, 2.00 mmol) and $FeCl_3$ (648 mg, 4.00 mmol) gave a pale yellow oil. Purification by flash chromatography (1.5 x 15 cm, EtOAc) gave **56** (42.6 mg, 31%): bp 185-190 °C at 0.7 mm Hg; IR ($CH_2Cl_2$ cast ) 3307 (br), 1738, 1651, 1544, 1438, 1210 $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3$) δ 6.14 (b s, 1H, N**H**), 5.67-5.55 (m, 2H, C**H**=C**H**), 4.26 (b s, 1H, CHO**H**), 3.85-3.80 (m, 2H, $CH_2$NHC(O)), 3.78 (s, 3H, CH(OH)$CO_2CH_3$), 3.69 (s, 3H, $CO_2CH_3$), 3.33 (b s, 1H, O**H**), 2.68 (t, 2H, *J* = 6.8 Hz, succinyl $CH_2$), 2.58-2.35 (m, 2H, CH(OH)$CH_2$), 2.49 (t, 2H, *J* = 6.8 Hz, succinyl $CH_2$); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) δ 174.1, 173.1, 171.2, 129.5, 126.3, 69.8, 51.8, 51.3, 40.7, 36.7, 30.2, 28.6; exact mass 273.1214 (273.1212 calcd for $C_{12}H_{19}NO_6$). Anal. Calcd for $C_{12}H_{19}NO_6$: C, 52.74; H, 7.01; N, 5.13. Found: C, 52.49; H, 7.17; N, 5.17.

**Methyl *N*-(4'-methylsuccinyl)-6-amino-2-hydroxyhexanoate ¶ (57)**

A solution of **56** (580 mg, 2.12 mmol) in MeOH (25 mL) was treated with 5% Pd on carbon (150 mg) and stirred under an atmosphere of hydrogen for 2.5 h. The catalyst was removed by filtration through Celite, and the solvent was removed *in vacuo* to give a pale yellow oil. Drying under high vacuum afforded **57** (561 mg, 96%). A sample was distilled *in vacuo* (bulb to bulb) for characterization: bp 190-200 °C at 0.4 mm Hg; IR ($CHCl_3$ cast) 3300 (br), 2952, 1738, 1652, 1551, 1438, 1047 $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3$) δ 6.33 (b s, 1H, N**H**), 4.23-4.17 (m, 1H, C**H**OH), 3.77 (s, 3H, CH(OH)$CO_2CH_3$), 3.68 (s, 3H, $CO_2CH_3$), 3.47 (d, 1H, *J* = 5.7 Hz, O**H**), 3.24 (dt, 2H, *J* = 6.7, 6.1 Hz, $CH_2$NHC(O)), 2.66 (t, 2H, *J* = 6.8 Hz, succinyl $CH_2$), 2.48 (t, 2H, *J* = 6.8 Hz, succinyl $CH_2$), 1.82-1.39 (b m, 6H, 3 x $CH_2$); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) δ 175.3, 173.4, 171.4, 70.1, 52.2, 51.6, 39.1, 33.6, 30.7, 29.2, 28.9, 21.9;

exact mass 275.1364 (275.1369 calcd for $C_{12}H_{21}NO_6$). Anal. Calcd for $C_{12}H_{21}NO_6 \cdot 1/2H_2O$: C, 50.70; H, 7.80; N, 4.93. Found: C, 51.21; H, 7.61; N, 5.00.

**Methyl *N*-(4'-methylsuccinyl)-6-amino-2-oxohexanoate [§] (58)**

Compound **58** was prepared by the method used for **49**. Thus reaction of **57** (561 mg, 2.04 mmol) with the Dess-Martin periodinane (1.73 g, 4.08 mmol) in $CH_2Cl_2$ (25 mL) for 1.5 h gave a pale yellow oil after work-up. Purification by flash chromatography (EtOAc) gave **58** (87.4 mg, 16%) as a white solid. A sample was recrystallized from EtOAc/ hexanes for characterization: mp 61-66 °C; IR ($CHCl_3$ cast) 3310, 2755, 1733, 1650, 1550, 1440, 1170 $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3$) δ 5.76 (b s, 1H, N**H**), 3.87 (s, 3H, C(O)$CO_2CH_3$), 3.69 (s, 3H, $CO_2CH_3$), 3.27 (dt, 2H, *J* = 6.9, 6.1 Hz, $CH_2$NHC(O)), 2.89 (t, 2H, *J* = 6.9 Hz, C(O)$CH_2$), 2.68 (t, 2H, *J* = 6.8 Hz, succinyl $CH_2$), 2.47 (t, 2H, *J* = 6.8 Hz, succinyl $CH_2$), 1.70-1.50 (m, 4H, 2 x $CH_2$); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) δ 193.9 (**C**(O)$CO_2CH_3$), 173.5 ($CH_2CH_2CO_2CH_3$), 171.4 (**C**(O)NH), 161.4 (C(O)$CO_2CH_3$), 52.9 (C(O)$CO_2CH_3$), 51.8 ($CO_2CH_3$), 39.0 ($CH_2$NH), 38.8 ($CH_2$C(O)$CO_2$), 31.1 ($CH_2$C(O)NH), 29.4 ($CH_2CH_2$NH), 28.8 ($CH_2CO_2CH_3$), 20.0 ($CH_2CH_2$C(O)$CO_2$); exact mass 273.1213 (273.1212 calcd for $C_{12}H_{19}NO_6$).

**Activity Assay for *N*-Succinyl DAP Aminotransferase**

Enzyme assays were run over 5-10 min at 30 °C in a volume of 1.0 mL, and the decrease in absorbance at 336-344 nm, due to oxidation of NADPH, was observed with a reference range at 500-520 nm. The assay mixture consisted of 100 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM EDTA.4Na, 50 mM L-glutamic acid, 100 mM $NH_4Cl$, bovine serum albumin (1 mg/ml), 5 mM $NaN_3$, 0.25 mM PLP, 0.15 mM NADPH, glutamate dehydrogenase (E.C. 1.4.1.4, 10 Units), 5 mM (6*S*)-6-*N*-succinylamino-2-oxo-heptan-1,7-dioic acid, tri-lithium salt (**50**), and enzyme solution (sufficient to allow $\Delta A_{340}$ of 0.2-1.5 units over the period of the assay). A stock solution (adjusted to pH 8.0) containing

the first 5 ingredients was kept at 4 °C for up to a month. A working stock solution, with PLP and NADPH added, was made up on a daily basis and kept at 30 °C while in use. The enzyme solutions were added individually to each assay. The spectrophotometer was zeroed on a mixture of the working solution (970 µL) plus the two enzyme solutions (10 µL each) in a 1 mL semi-micro quartz cuvette with a path length of 1 cm. This mixture was incubated at 30 °C for 3 min before the assay was initiated by the addition of the α-keto substrate (**50**, 10-50 µL). Initial rates were determined from the linear sections of the absorbance curve excluding any initial lag phase. In the case of a continous concave curve the rate was determined for the first 30 seconds. Enzyme activity in Units (µmoles substrate converted per min) was related to $\Delta A_{340}$/min by the formula:

$$\text{Activity} = \frac{[\Delta A/\text{min}] \times V_{assay} /}{\varepsilon \times l} \times [V_{tot}/V_{enz}] \times 1000$$

where: $\Delta A/\text{min}$ = maximum decrease in absorbance at 340 nm per minute

$V_{assay}$ = assay volume in mL (1 mL)

$V_{tot}$ = total volume of enzyme solution in mL

$V_{enz}$ = volume of enzyme solution used in the assay, in mL

$\varepsilon$ = extinction coefficient for NADPH at 340 nm. ($6.2 \times 10^3$ L/mol.cm)

$V_{enz}$ = volume of enzyme solution used (usually 10 µL)

$l$ = light path in cm (1 cm)

The factor of 1000 arises because the enzyme Units are in $\mu mol.min^{-1}$, whereas the extinction coefficient has units of L/mol·cm, and the assay volume is in mL. The specific activity of the enzyme solution (enzyme activity/unit protein) is expressed as enzyme Units / mg protein.

Controls were run excluding one assay ingredient at a time. No activity was observed when any of the following were excluded: either enzyme, the α-keto substrate (**50**), L-glutamic acid, or NADPH.

**Determination of Protein Concentration**

Protein concentrations were determined with the BioRad protein assay dye reagent (Coomassie Blue, Bradford method[177]) using a modification of the recommended assay procedure. Standard curves (μg/μL) were prepared using an aqueous solution of bovine serum albumin (1.00 mg/mL), assaying 10 samples between 0 and 40 μL (0-40 μg). The BioRad reagent (2 mL, 5-fold dilution) was added to each sample in a 3 mL cuvette and mixed. After being incubated for 15 min at room temperature, the samples were assayed at 596 nm against a reagent blank. The dilution due to different sample size (<40 μL, <2%) was treated as insignificant.

**Aminotransferase Isolation**

All procedures were performed at 4 °C unless otherwise stated. The results are summarized in Table **6** in the Results and Discussion section. Activity assays for the enzyme isolation were performed at 30 °C and pH 8.0 with the following composition: 100 mM $NH_4Cl$, 50 mM L-glutamic acid, 0.25 mM PLP, 0.1 mM NADPH, 1 mM α-keto substrate (**50**), 0.25 Units of glutamate dehydrogenase, enzyme solution (10-100 μL), and 200mM Tris-HCl. The assay volume was 1.0 mL.

1. **Crude Extract**

The aminotransferase was isolated from *E.coli W* (ATCC 9637, cultured in the Department of Microbiology, University of Alberta). The cells were grown in Davis and Mingioli medium,[205] supplemented with yeast extract (3 g/L), peptone (5 g/L), and glucose (5 g/L). A 10 L culture was harvested in late log phase. Cells were filtered, washed with 30 mM phosphate buffer, pH 7.2, and centrifuged (Sorvall RC5B centrifuge, GSA rotor), to give a total wet weight of approximately 120 g. The pellet was resuspended in 20 mM MOPS, pH 8.0 (130 mL with 10 μg/mL PLP). The cells were broken by sonication in a continous flow sonicator (3 x 1 min passages) chilled to -16 °C. Cell debris was removed by centrifugation (Sorvall SS34 rotor; 1 h at 14,000 rpm) and the supernatant was frozen at -20 °C.

2. **$MnCl_2$ Precipitation**

The procedure of Peterkofsky and Gilvarg[70] was followed. A solution of 1 M $MnCl_2$ (6 mL, final concentration = 50 mM) in 20 mM MOPS, pH 8.0, was added slowly dropwise to the thawed cell-free extract, with stirring at 0 °C. A mixture of protease inhibitors (1.0 mM PMSF, 1 μM Pepstatin A, 1 mM EDTA) was also added. The mixture was stirred for 15 min then allowed to stand for 2 h. The precipitate was removed by centrifugation (GSA rotor, 30 min at 10,000 rpm) and the supernatant was dialysed overnight against 10mM Tris, pH 8.0, (3 x 2 L) containing PLP (10 μg/mL) and protease inhibitors (including 0.2 mM PMSF). The resulting dialysate (120 mL) was centrifuged (20 min at 10,000 rpm) to remove precipitated protein. Measurement of protein and activity levels indicated there was 2.30 g of protein and 1,570 Units of enzyme activity.present.

3. **Ion Exchange Chromatography**

The dialysate was applied to a column of ion exchange cellulose (Whatman DE-52, 5 x 20 cm, equilibrated in 10 mM Tris-HCl, pH 8) at 2.5 mL/min. The enzyme was eluted with a KCl gradient of 0 - 0.5 M in the same buffer, followed by isocratic elution with 0.5 M KCl, with protein detection at 280 nm, collecting 25 mL fractions. Fractions were assayed for protein and activity. Those fractions with high activity were combined and concentrated by ultrafiltration to 10 mL total volume. Glycerol was added (25%) and the material was frozen at -20 °C. Protein content was 570 mg with a total activity of 442 Units. The less active fractions were similarly treated. This material contained 352 mg of protein and 171 Units of enzyme activity.

4. **Sephadex G-100 Chromatography**

The higher-activity material from the previous column was purified on a Sephadex G -100 column (2.5 x 89 cm) in 7 batches of 2 mL each. The columns were run in 50 mM Tris-HCl / 150 mM KCl / 0.1 mM EDTA, pH 8, at 30 mL/h with detection at 218 nm, collecting 5 mL fractions. The major activity eluted between 160-195 mL. All active fractions were

combined and concentrated by ultrafiltration to a final volume of 6.5 mL. Glycerol (2 mL) and PLP (10 µg/mL) were added and the solution was frozen at -20 °C. The protein content was 205 mg and the activity was 75.4 Units. Concentration of lower activity fractions provided a further 87 mg of protein and 23.5 mU of activity. Due to the broad nature of the activity peak no estimation of the molecular weight for the native enzyme was made from this column.

5. **Dye-Ligand Affinity Chromatography**

Binding Studies. Five agarose-bound dyes were tested for enzyme binding: Cibacron Blue 3G-A-Agarose Type 3000-CL, Reactive Brown-10, Reactive Green-19, Reactive Yellow-86, and Reactive Blue-4 (all obtained from Sigma). Small columns with 1 mL (0.20 g) of each dye were prepared in pasteur pipettes in 50mM Tris, pH 7.5. Enzyme solution (the less active material from the DE-52 column) was exchanged into the same buffer (Amicon Centriprep-10), and 0.75 mL (20 mg protein) was applied to each column. The columns were washed with buffer and then eluted with 1 M NaCl (in the same buffer). Protein and activity levels were determined for the column wash and eluant fractions. The results are shown in Figure **16** in the Results and Discussion section. The two strongest enzyme-binding columns, Blue-3G-A and Reactive Green-19 (or the equivalent Amicon Matrex-Green), were tested in a similar manner for *N*-succinyl DAP aminotransferase-binding strength, at two different pH values, with, and without, the inclusion of 5 mM $MgCl_2$. The buffers used were 50 mM MES, pH 6.0, and 50 mM Tris-HCl, pH 8.0. Strong binding was observed at pH 6 while very little binding occurred at pH 8. The inclusion of $Mg^{2+}$ had no observable effect on the binding. The enzyme-bound columns were then tested for specific elution with 30 mM DAP (commercial mixture of isomers), followed by 20 mM *N*-succinyl DAP (L,L and D,D isomers) in the same buffer. No significant amount of enzyme was eluted with either step. The aminotransferase was then eluted with 50 mM Tris-HCl, pH 8.0. Higher recovery of activity was observed from the blue column than the green column. In the next step the pH limit for enzyme binding and recovery in

different buffers was determined for the blue dye. Enzyme samples were bound in 50 mM MES pH 6.0 then eluted with pH steps of 0.5 units with either 50 mM MOPS (to pH 8), 30m M phosphate (to pH 8), or 50 mM Tris-HCl (to pH 9). Loss of binding began at pH 7.0 in phosphate and pH 7.5 in MOPS and Tris-HCl. Stronger binding at lower pH values was observed with phosphate, as well as greater recovery of activity at higher pH, compared to the other buffers. The concentration of NaCl required for elution of activity from the blue column was also determined. Enzyme samples were bound in either 30 mM phosphate, pH 6.5, or in 50 mM MOPS, pH 7.5, and then eluted with 0.1-0.5 N NaCl in 0.1 N steps. All activity came off the columns with 0.1-0.2 N NaCl. Higher recovery of activity was again achieved with the phosphate buffer. The binding conditions were then tested on the Reactive Brown-10 dye. An enzyme sample (0.5 mL, 20 mg protein) was applied to a 1.5 mL column of the dye in 30 mM phosphate, pH 6.5, and eluted with a stepwise pH gradient to pH 8.0, followed by elution with 2 N NaCl. The column initially bound 25% of the total protein and 20% of the activity; the latter required 2 N NaCl for elution from the column.

Full-Scale Purification: The higher-activity enzyme solution from the Sephadex column was exchanged into 30 mM phosphate, pH 6.5, (Amicon Centriprep-10) and applied to a column of Reactive Brown-10-agarose (29 mL, 2.5 cm i.d.) connected in series with a column of Cibacron Blue 3G-A-Agarose Type 3000-CL (50 mL, 2.5 cm i.d.) at 0.5 mL/min, collecting 10 mL fractions, and with detection at 280 nm. Once the non-binding protein had eluted from the blue column, the columns were disconnected and eluted separately. The blue column was initially eluted with a pH gradient from pH 6.5-7.5 in 30 mM phosphate at 1 ml/min, followed by isocratic elution at pH 8, and then eluted sequentially with 0.1 N, 0.2 N, and 1.0 N NaCl in 30 mM phosphate, pH 8. The brown column was washed off with 30 mM phosphate, pH 8.5, followed by 1.0 N NaCl in the same buffer, eluting 57 mg of protein which contained a small amount of activity. The majority of the activity was eluted from the blue column between pH 7.5 and 8.0. A small

amount of activity eluted with 0.1-0.2 N NaCl. The most active fractions were combined and concentrated by ultrafiltration to 5 mL. Lower activity fractions were similarly treated. The concentrated samples were frozen at -60 °C with added glycerol (10%) and PLP (10 µg/mL). The higher activity material contained 58.3 Units of activity and 3.6 mg protein. The less active material contained 50.9 Units of activity and 5.5 mg of protein.

6. **Hydroxy-Apatite Chromatography**

The higher activity material from the previous column was applied to a column of hydroxy-apatite (BioRad HTP-Gel, 1 x 5 cm, 4 mL) packed in 1 mM phosphate, pH 6.8, with added PLP (10µg/mL), at 25 mL/h collecting 2.5 mL fractions. The column was washed with buffer (25 mL), followed by 10 mM phosphate, pH 6.8, and then eluted with a phosphate gradient from 10-300 mM. Elution was continued with 0.3 N and then 0.5 N NaCl. A large protein band eluted immediately prior to the activity at 0.2-0.3 N NaCl. The activity recovered was 43.8 Units and 1.48 mg of protein, giving a specific activity of 29.6 Units/mg. The most active fraction had a specific activity of 60.5 Units/mg.

**Determination of $K_m$ and $V_{max}$**

The Michaelis-Menten constants were determined on the most active fraction from the hydroxy-apatite column (specific activity of 60.5 Units/mg, see Figure **17b** for the SDS-PAGE gel) which had been frozen in 0.2 M phosphate, pH 6.8, (10% glycerol) at -60 °C. Initial rate data were obtained from assays run as previously described, using 10 Units of glutamate dehydrogenase per assay, and 1 µL of the enzyme solution, at 7 different α-keto substrate (**50**) concentrations which were repeated at 5 different glutamate concentrations, for a total of 35 rate measurements. $K_m^{app}$ and $V_{max}^{app}$ values were determined for each glutamate concentration by computer, using non-linear regression analysis (Enzyme Kinetics, Trinity Software, Campton, NH). Values of $K_m$ for both substrates, and $V_{max}$, were determined in the same way; by plotting $K_m^{app}$ and $V_{max}^{app}$ values against glutamate

and α-keto substrate concentrations. The calculated values are given in Table **7** in the Results and Discussion Section.

**Inhibition Studies**

These studies were performed by Dr. Russell J. Cox. Assays were conducted using the previously described procedure at the same enzyme concentrations as used for determination of the kinetic constants. Inhibitors were added to the assay mixture prior to addition of the α-keto substrate, at 30 °C, and the baseline absorbance levels were monitored to observe any turnover of the inhibitor (or other change). Upon initiation of the assay by addition of the α-keto substrate the absorbance level was monitored for 10 min. The activity and inhibition results for the desamino (**5**) and descarboxy (**6**) analogs, as well as the tetrahydrodipicolinate analog, α-hydroxy-tetrahydropyrandicarboxylate[68] (2-HTHP), are given in Table **8** in the Results and Discussion section. Controls run with no aminotransferase present showed no activity. The assay composition was modified slightly for the *N*-amino-analog (**3**), hydrazine, and hydroxylamine, which react with exogeneous PLP in the assay mixture to cause an increase in absorbance at 340 nm. Therefore, no PLP was added to the assay mixture. The PLP present in the amino-transferase solution resulted in a final PLP concentration in the assay of 3.4 μM. Compound **3**, hydroxylamine, and hydrazine, were assayed for inhibition at three concentrations: 1.0, 0.1, and 0.01 mM. The results are presented in Table **9** in the Results and Discussion section.

## REFERENCES


1. O'Donnell, M.J. (Ed.) *Tetrahedron* **1988**, *44*, 5253-5614.
2. Williams, R.M. *Synthesis of Optically Active α-Amino Acids*; Pergamon Press: Oxford, 1989.
3. Coppola, G.M.; Schuster, H.F. *Asymmetric Synthesis: Construction of Chiral Molecules Using Amino Acids*; John Wiley: New York, 1987.
4. Barrett, G.C. *Chemistry and Biochemistry of the Amino Acids;* Chapman and Hall: London, 1985.
5. Greenstein, J.P.; Winitz, M. *Chemistry of the Amino Acids*; Vol 1-3; Robert E. Krieger: Malabar, Florida, 1984.
6. Belokon, Y.N.; Bakhmutov, N.I.; Chernoglazova, N.I.; Kochetkov, K.A.; Vitt, S.V.; Garbalinskaya, N.S. ; Belikov, V.M. *J. Chem. Soc. Perkin Trans. I* **1988**, 305-312.
7. Evans, D.A; Britton, T.C.; Dorow, R.L.; Dellaria, J.F. *J. Amer. Chem. Soc.* **1986**, *108*, 6395-6397.
8. Trimble, L.A.; Vederas, J.C. *J. Amer. Chem. Soc.* **1986**, *108*, 6397-6399.
9. Evans, D.A.; Britton, T.C. *J. Amer. Chem. Soc.* **1987**, *109*, 6881-6883.
10. Paul, P.K.C.; Sukumar, M.; Bardi, R.; Piazzesi, A.M.; Valle, G.; Toniolo, C.; Balaram, P. *J. Am. Chem. Soc.* **1986**, *108*, 6363-6370.
11. Saari, W.S.; Freedman, M.B.; Hartman, R.D.; King, S.W.; Raab, A.W.; Randall, W.C.; Engelhardt, E.L.; Hirschmann, R.; Rosegay, A.; Ludden, C.T.; Scriabine, A. *J. Med. Chem.* **1978**, *21*, 746-753.
12. Saari, W.; Halczenko, W.; Cochran, D.W.; Dobrinska, M.R.; Vincek, W.C.; Titus, D.G.; Gaul, S.L.; Sweet, C.S. *J. Med. Chem.* **1984**, *27*, 713-717.
13. Ramalingam, K.; Woodward, R.W. *Tetrahedron Lett.* **1985**, *26*, 1135-1136.

14. Walsh, J.J.; Metzler, D.E.; Powell, D.; Jacobson, R.A. *J. Am. Chem. Soc.* **1980**, *102*, 7136-7138.

15. Bugg, T.D.H.; Walsh, C.T. *Nat. Prod. Rep.* **1992**, *9*, 199-215.

16. Ward, J. B. In *Antibiotic Inhibitors of Bacterial Cell Wall Biosynthesis*; Tipper, D. J., Ed.; Pergamon Press: New York, 1987; pp 1-43.

17. Rogers, H. J.; Perkins, H. R.; Ward, J. B. In *Microbial Cell Walls and Membranes*; Chapman and Hall: London, 1980; pp 191-214.

18. Ghuysen, J. M.; Frere, J. M.; Joris, B.; Dusant, J.; Duez, C.; Leyh-Bouille, M.; Nguyen-Disteche, M.; Coyette, J.; Dideberg, O.; Charlier, P.; Dive, G.; Lamotte-Brasseur, J. In *Design of Enzyme Inhibitors as Drugs*; Sandler, M.; Smith, H. J., Ed.; Oxford Science Publications: London, 1989; pp 523-572.

19. Bricas, E.; Ghuysen, J.M.; Dezelee, P. *Biochemistry* **1967**, *6*, 2598-2606.

20. Wickus, G. G.; Strominger, J. L. *J. Biol. Chem.* **1972**, *247*, 5297-5306.

21. Saleh, F.; White, P. *J. Gen . Microbiol.* **1979**, *115*, 95-100.

22. DasGupta, H.; Fan, D. P. *J. Biol. Chem.* **1979**, *254*, 5672-5683.

23. Takahashi, Y.; Iwai, Y.; Tomoda, H.; Nimura, N.; Kinoshita, T.; Omura, S. *J. Gen. Appl. Microbiol.* **1989**, *35*, 27-32.

24. Mengin-Lecreulx, D.; Michaud, C.; Richaud, C.; Blanot, D.; Van Heijenoort, J. *J. Bacteriol.* **1988**, *170*, 2031-2039.

25. Kawamoto, I.; Oka, T.; Nara, T. *J. Bacteriol.* **1981**, *146*, 527-534.

26. Perkins, H. R. *Biochem. J.* **1969**, *115*, 797-805.

27. Fredriksen, A.; Vasstrand, E. N.; Jensen, H. B. *J. Bacteriol.* **1991**, *173*, 900-902.

28. Glauner, B.; Holtje, J.-V.; Schwarz, U. *J. Biol. Chem.* **1988**, *263*, 10088-10095.

29. Braun, V.; Rehn, K. *Eur. J. Biochem.* **1969**, *10*, 426-438.

30. Mirelman, D. In *Bacterial Outer Membranes: Biogenesis and Functions*; Inouye, M., Ed.; John Wiley and Sons: New York, 1979; pp 115-166.

31. Johannsen, L.; Wecke, J.; Obal. F.Jr.; Krueter, J.M. *Amer. J. Physiology* **1991**, *260*, R126-R133.

32. Luker, K.E.; Collier, J.L.; Kolodziej, E.W.; Marshall, G.R.; Goldman, W.E. *Proc. Natl Acad. Sci. USA* **1993**, *90*, 2365-2369.

33. Sinha, R.K.; Rosenthal, R.S. *Infect. Immun.* **1980**, *29*, 914-925.

34. Sen, Z.; Karnovsky, M.L. *Infect. Immun.* **1984**, *43*, 937-941.

35. Pappenheimer, J.R.; Koski, G.; Fencl, V.; Karnovsky, M.L.; Krueger, J. *J. Neurophysiol.* **1975**, *38*, 1299-1550.

36. Gotoh, T.; Nakahara, K.; Nishiura, T.; Hahimoto, S.; Kino, T.; Kuroda, Y. *J. Antibiot.* **1982**, *35*, 1286-1292.

37. Mine,Y.; Watanabe, Y.; Tawara, S.; Yokota, Y.; Nishida, N.; Goto, S.; Kuwahara, S. *J. Antibiot.* **1983**, *30*, 1059-1066.

38. Oku, T.; Imanishi, J.; Kishida, T. *Antiviral Res.* **1986**, *6*, 733-739.

39. Yi-Li, W.; Kaplan, S.; Whiteside, T.; Heberman, R.H. *Immunopharmacology* **1989**, *18*, 213-222.

40. Talmadge, J.E.; Lenz, B.; Schneider, M.; Phillips, H.; Long, C. *Cancer Immunol. Immunother.* **1989**, *28*, 93-100.

41 Walter, J.; Davenne, D.; Shoham, S.; Dinarello, C.A.; Krueger, J.M. *Am. J. Physiol.* **1986**, *250*, R96-R103.

42. Bahr, G.M.; Chedid, L.A.; Behbehani, K. *Cell. Immunol.* **1987**, *107*, 443-454.

43. Ahmed, K.; Turk, J.L.*Cancer Immunol. Immunother.* **1989**, *28*, 87-92.

44 Work, E.; Birnbaum, S.M.; Winitz, M.; Greenstein, J.P. *J. Am. Chem. Soc.* **1955**, *77*, 1916-1918.

45. Wade, R.; Birnbaum, S. M.; Winitz, M.; Koegel, R. J.; Greenstein, J. P. *J. Am Chem. Soc.* **1957**, *79*, 648-652.

46. Van Heijenoort, J.; Bricas, E. *Bull. Soc. Chim. Fr.* **1968**, 2828-2831.

47. Izumi, Y. *J. Chem. Soc. Jpn* **1954**, *75*, 1152-1155.

48. Arendt, A.; Kolodziejczyk, A. Sokolowska, T. Szufler, E. *Roczniki Chemii* **1974**, *48*, 635-639.

49. Bouchadon, J.; Dutruc-Rosset, G.; Farge, D.; James, C. *J. Chem. Soc. Perkin Trans. I* **1989**, 695-701.

50. Bricas, E.; Ghuysen, J.-M.; Dezelee, P. *Biochemistry* **1967**, *6*, 2598-2606.

51. Dezelee, P.; Bricas, E. *Biochemistry* **1970**, *9*, 823-831.

52. Hemmi, K.; Takeno, H.; Okada, S.; Nakaguchi, O.; Kitaura, Y.; Hashimoto, M *Tetrahedron Lett.* **1982**, *23*, 693-696.

53. Kolodzieczyk , A.M.; Kolodzieczyk, A.S.; Stoev, S. *Int. J. Peptide Protein Res.* **1992**, *39*, 382-387.

54. . Patte, J.-C. In *Amino Acids; Biosynthesis and Genetic Regulation*; Herrmann, K. M.; Somerville, R.L., Ed.; Addison-Wesley: Reading, MA, 1983; pp 213-228.

55. Yeh, P.; Sicard, A.M.; Sinskey, A.J. *Mol. Gen. Genet* **1988**, *212*, 105-111.

56. Rosenthal, G.A. *Plant Nonprotein Amino and Imino Acids; Biological, Biochemical, and Toxicological Properties*; Academic Press: New York, 1982; pp 168-171.

57. Tyagi, V.V.S.; Henke, R.R.; Farkas, W.R. *Biochim. Biophys. Acta* **1982**, *719*, 363-369.

58. Mazelis, M.; Creveling, R.K. *J. Food Sci.* **1978**, *2*, 29-37.

59. Sodek, L. *Phytochemistry* **1976**, *15*, 1903-1906.

60. Kato, Y. *Mem. Tokyo Univ. Agric.* **1979**, *21*, 57-96 (C.A. 92:211880z).

61. Dougall, D.K.; Fulton, M.M. *Plant Physiol.* **1967**, *42*, 941-945.

62. Moller, B.L. *Plant Physiol.* **1974**, *54*, 638-643.

63. Wenko, L.K.; Treick, R.W.; Wilson, K.G. *Plant Mol. Biol.* **1985**, *4*, 197-204.

64. Wink, M.; Hartmann, T. *Plant Physiol.* **1982**, *70*, 74-77.

65. Mills, W.R.; Lea, P.J.; Miflin, B.J. *Plant Physiol.* **1980**, *65*, 1166-1172.

66. Mazelis, M.; Miflin, B.J.; Pratt, H.M. *FEBS Lett.* **1976**, *64*, 197-200.

67. Gilvarg, C. *J. Biol. Chem.* **1961**, *236*, 1429-1431.

68. Berges, D.A.; DeWolf, W.E., Jr.; Dunn, G.L.; Newman, D.J.; Schmidt, S.J.; Taggart, J.J.; Gilvarg, C. *J. Biol. Chem.* **1986**, *261*, 6160-6167.

69. Wiseman, J.S.; Nichols, J.S. *J. Biol. Chem.* **1984**, *259*, 8907-8914.

70. Peterkofsky, B.; Gilvarg, C. *J. Biol. Chem.* **1961**, *236*, 1432-1438.

71. Peterkofsky, B. *Methods Enzymol.* **1962**, *5*, 853-858.

72. Lin, Y.; Myhrman, R.; Schrag, M.L.; Gelb, M.H. *J. Biol. Chem.* **1988**, *263*, 1622-1627.

73. Kindler, S. H.; Gilvarg, C. *J. Biol. Chem.* **1960**, *235*, 3532-3535.

74. Antia, M.; Hoare, D.S.; Work, E. *Biochem. J.* **1957**, *65*, 448-459.

75. Higgins, W.; Tardif, C.; Richaud, C.; Krivanek, M.A.; Cardin, A. *Eur. J. Biochem.* **1989**, *186*, 137-143.

76. Work, E. *Methods Enzymol.* **1962**, *5*, 864-870.

77. Asada, Y.; Tanizawa, K.; Kawabata, Y.; Misono, H.; Soda, K. *Agric. Biol. Chem.* **1981**, *45*, 1513-1514.

78. Kelland, J.G.; Palcic, M.M.; Pickard, M.A.; Vederas, J.C. *Biochemistry* **1985**, *24*, 3263-3267.

79. Asada, Y.; Tanizawa, K.; Sawada, S.; Suzuki, T.; Misono, H.; Soda, K. *Biochemistry* **1981**, *20*, 6881-6886.

80. Misono, H.; Ogasawara, M.; Nagasaki, S. *Agric. Biol. Chem.* **1986**, *50*, 1329-1330.

81. Ishino, S.; Yamaguchi, K.; Shirahata, K.; Araki, K. *Agric. Biol. Chem.* **1984**, *48*, 2557-2560.

82. Misono, H.; Ogasawara, M.; Nagasaki, S. *Agric. Biol. Chem.* **1986**, *50*, 2729-2734.

83. Bartlett, A.T.M.; White, P.J. *J. Gen. Microbiol.* **1985**, *131*, 2145-2152.

84. White, P.J. *J. Gen. Microbiol.* **1983**, *129*, 739-749.

85. Misono, H.; Togawa, H.; Yamamoto, T.; Soda, K. *J. Bacteriol.* **1979**, *137*, 22-27.

86. Misono, H.; Soda, K. *J. Biol. Chem.* **1980**, *255*, 10599-10605.

87. Schrumpf, B.; Schwarzer, A.; Kalinowski, J.; Pühler, A.; Eggeling, L.; Sahm, H. *J. Bacteriol.* **1991**, *173*, 4510-4516.

88. Bouvier, J.; Richaud, C.; Higgins, W.; Bögler, O.; Stragier, P. *J. Bacteriol.* **1992**, *174*, 5265-5271; and references cited therein.

89. Christen, P.; Metzler, D.E. (Ed.) *Transaminases*; John Wiley and Sons: New York, 1985.

90. Braunstein, A.E. In *The Enzymes* , Volume 9; Boyer, P.D., Ed.; third edition, 1973; p. 378-481.

91. Snell, E.E.; DiMari, S.J. *The Enzymes* **1970**, *2*, 335-370.

92. Fasella, P.; Turano, P. *Vitam. Horm.* **1970**, *28*, 157-194.

93. Dunathan, H.C. *Adv. Enzymol.* **1971**, *35*, 79-134.

94. Wroblewski, F. *Adv. Clin. Chem.* **1958**, *1*, 313-351.

95. Salerno, C.; Giartosio, A.; Fasella, P. In *Vitamin B6 Pyridoxal Phosphate Part B.* Chapter 5 (Transaminases); Dolphin, D.; Poulson, R.; Avramovic, O., Ed.; John Wiley and Sons: NewYork, 1986.

96. Torchinsky, Y.M. In *Vitamin B6 Pyridoxal Phosphate Part B.* Chapter 6 (Aspartate Aminotransferase); Dolphin, D.; Poulson, R.; Avramovic, O., Ed.; John Wiley and Sons: NewYork, 1986.

97. Cooper, A.J.L.; Griffith, O.W. *J. Biol. Chem.* **1979**, *254*, 2748-2753.

98. Yamada, R.-H.; Wakabayashi, Y.; Iwashima, A.; Hasegawa, T. *Biochim. Biophys. Acta* **1984**, *801*, 151-154.

99. Scaman, C.H.; Palcic, M.M.; McPhalen, C.; Gore, M.K.; Lam, L.K.P.; Vederas, J.C. *J. Biol. Chem.* **1991**, *266*, 5525-5533.

100. Inoue, H.; Kato, Y.; Takigawa, M.; Adachi, K.; Takeda, Y. *J. Biochem.* **1975**, *77*, 879-893.

101. Kato, Y. Inoue, H.; Goda, E.; Tamada, F.; Takeda, Y. *Gann* **1976**, *67*, 569-576 (C.A. 85:137404).

102. Harik, S.I.; Snyder, S.H. *Biochim. Biophys. Acta* **1973**, *327*, 501-509.

103. Levine, R.J.; Sato, T.L.; Sjoerdsma, A. *Biochem. Pharmacol.* **1965**, *14*, 139-149.

104. Maslinski, C.; Niedzielski, A. *Eur. J. Pharmacol.* **1969**, *5*, 196-202.

105. Tanase, S.; Guirard, B.M.; Snell, E.E. *J. Biol. Chem.* **1985**, *260*, 6738-6746.

106. Glamkowski, E.J.; Gal, G.; Sletzinger, M.; Porter, C.C.; Watson, L.S. *J. Med. Chem.* **1967**, *10*, 852-855.

107. Waterhouse, M.J.; Chia, Y.C.; Lees, G.J. *Mol. Pharmacol.* **1979**, *15*, 108-114.

108. Creveling, C.R.; Daly, J.W.; Witkop, B. *J. Med. Chem.* **1966**, *9*, 284-286.

109. Van Gelder, N.M. *J. Neurochem.* **1968**, *15*, 747-757.

110. Van Gelder, N.M. *J. Neurochem.* **1969**, *16*, 1355-1360.

111. Lam, L.K.P.; Arnold, L.D.; Kalantar, T.H.; Kelland, J.G.; Lane-Bell, P.M.; Palcic, M.M.; Pickard, M.A.; Vederas, J.C. *J. Biol. Chem.* **1988**, *263*, 11814-11819.

112 Ahmad,S.; Alauddin, M.; Caddy, B.; Martin-Smith, M.; Sidwell, W.T.L.; Watson, T.R. *Aust. J. Chem.* **1971**, *24*, 521-547.

113. Wessely, F.; Swoboda, W. *Monatsh. Chem.* **1951**, *82*, 437-442.

114. Spero, G.B.; McIntosh, A.V. Jr.; Levin, R.H. *J. Am. Chem. Soc.* **1948**, *70*, 1907-1909.

115. Barton, D.H.R.; Faro, H.P.; Serebryakov, E.P.; Wooley, N.F. *J. Chem. Soc.* **1965**, 2438-2444.

116. Profilis, C.; Catsoulacos, P. *Eur. J. Med. Chem.- Chim. Ther.* **1983**, *18*, 567-568.

117. Lapatsanis, L.; Profilis, C.; Catsoulacos, P. *J. Chem. Eng. Data* **1980**, *25*, 287-289.

118. Diago-Meseguer, J.; Palomo-Coll, A.L. *Synthesis*, **1980**, 547-551.

119. Kamijo, T.; Harada, H.; Iizuka, K. *Chem. Pharm. Bull.* **1984**, *32*, 5044-5047.

120. Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591-596.

121. Prelog, V. *Helv. Chim. Acta* **1953**, *36*, 308-319.

122. Seebach, D. *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 1624-1654 and references cited therein.

123. Whitesell, J.K.; Lawrence, R.M.; Chen, H.H. *J. Org. Chem.* **1986**, *51*, 4779-4784.

124. Whitesell, J.K.; Chen, H.H.; Lawrence, R.M. *J. Org. Chem.* **1985**, *50*, 4663-4664.

125. Whitesell, J.K.; Nabona, K.; Deyo, D. *J. Org. Chem.* **1989**, *54*, 2258-2260.

126. Whitesell, J.K.; Carpenter, J.F.; Yaser, H.K.; Machajewski, T. *J. Am. Chem. Soc.* **1990**, *112*, 7653-7659.

127. Whitesell, J.K.; Allen, D.E. *J. Org. Chem.* **1985**, *50*, 3025-3026.

128. Whitesell, J.K.; Allen, D.E. *J. Am. Chem. Soc.* **1988**, *110*, 3585-3588.

129. Lane-Bell, P. PhD Thesis, University of Alberta, 1991

130. Whitesell, J.K.; Lawrence, R.M. *Chimia* **1986**, *40*, 318-321.

131. Leffler, J.E.; Tsuno, Y. *J. Org. Chem.* **1963**, *28*, 902-906.

132. Seebach, D.; Miller, D.D.; Muller, S.; Weber, T. *Helv. Chim. Acta* **1985**, *68*, 949-952.

133. Fitzi, R.; Seebach, D. *Tetrahedron* **1988**, *44*, 5277-5292.

134. Seebach, D.; Dziadulewicz, E.; Behrendt, L.; Cantoreggi, S.; Fitzi, R. *Liebigs Ann. Chem.* **1989**, 1215-1232.

135. Schickli, C.P.; Seebach, D. *Liebigs Ann. Chem.* **1991**, 655-668.

136. Seebach, D.; Burger, H.M.; Schickli, C.P. *Liebigs Ann. Chem.* **1991**, 669-684.

137. Seebach, D.; Juaristi, E.; Miller, D.D.; Schickli, C.P.; Weber, T. *Helv. Chim. Acta* **1987**, *70*, 237-261.

138. Gander-Coquoz, M.; Seebach, D. *Helv. Chim. Acta* **1988**, *71*, 224-236.

139. Schöllkopf, U.; Groth, U.; Gull, M.-R.; Nozulak, J. *Liebigs Ann. Chem.* **1983**, 1133-1151.

140. Schöllkopf, U. *Pure & Appl. Chem.* **1983**, *55*, 1799-1806.

141. Schöllkopf, U. *Topics Curr. Chem.* **1983**, *109*, 65-84.

142. Song, Y. Ph. D. Thesis, University of Alberta, 1991.

143. Mukhopadhay, T.; Seebach, D. Helv. Chim. Acta 1982, 65, 385-391.

144. Zanol, M.; Gastaldo, L. *J Chromatogr.* **1991**, *536*, 211-216.

145. Mengin-Lecreulx, D.; Michaud, C.; Richaud, C.; Blanot, D.; Van Heijenoort, J. *J. Bacteriol.* **1988**, *170*, 2031-2039.

146. Fitzi, R.; Seebach, D. *Angew. Chem. Int. Ed. Engl.* **1986**, *25*, 345-346; Correction: p766.

147. Bold, G.; Allmendinger, T.; Herold, P.; Moesch, L.; Schar, H.-P.; Duthaler, R.O. *Helv. Chim. Acta* **1992**, *75*, 865-882.

148. Jurgens, A.R. *Tetrahedron Lett.* **1992**, *33*, 4727-4730.

149. Garner, P.; Park, J.M. *Org. Syn.* **1992**, *70*, 18- 28

150. Williams, R.M.; Yuan, C. *J. Org. Chem.* **1992**, *57*, 6519-6527.

151. Baldwin, J.E.; Lee, V.; Schofield, C.J. *Synlett.* **1992**, 249-251.

152. Schöllkopf, U.; Hinrichs, R.; Lonsky, R. *Angew. Chem. Int. Ed. Engl.* **1987**, *26*, 143-145.

153. Williams, R.M.; Im, M.-N. *J. Am. Chem. Soc.* **1991**, *113*, 9276-9286.

154. Girodeau, J.-M.; Agouridas, C.; Masson, M.; Pineau, R.; Le Goffic, F. *J. Med. Chem.* **1986**, *29*, 1023-1030.

155. Agouridas, K; Girodeau, J.M.; Pineau, R. *Tetrahedron Lett.* **1985**, *26*, 3115-3118.

156. Kelly, T.R.; Schmidt, T.E.; Haggerty, J.G. *Synthesis* **1972**, 544-545.

157. Menger, F.M.; Lee, C. *J. Org. Chem.* **1979**, *44*, 3446-3448.

158. Dess, D.B.; Martin, J.C. *J. Org. Chem.* **1983**, *48*, 4155-4156.

159. Dess, D.B.; Martin, J.C. *J. Am. Chem. Soc.* **1991**, *113*, 7277-7287.

160. Ireland, R.E.; Liu, L. *J. Org. Chem.* **1993**, *58*, 2899.

161. Burkhart, J.P.; Peet, N.P.; Bey, P. *Tetrahedron Lett.* **1988**, *29*, 3433-3436.

162. Cooper, A.J.L.; Ginos, J.Z.; Meister, A. *Chem. Rev.* **1983**, *83*, 321-358.

163. Benigni, J.D.; Dickson, D.E. *J. Med. Chem.* **1966**, *9*, 439-440.

164. Takamura, N.; Yamada, S.-I. *Chem. Pharm. Bull.* **1976**, *24*, 800-803.

165. Brand, L.M.; Harper, A.E. *Biochemistry* **1976**, *15*, 1814-1821.

166. Borch, R.F.; Bernstein, M.D.; Durst, H.D. *J. Am. Chem. Soc.* **1971**, *93*, 2897-2904.

167. Ahmad, A. *Bull. Chem. Soc. Jap.* **1974**, *47*, 1819-1820.

168. Ahmad, A.; Spenser, I.D. *Can. J. Chem.* **1961**, *39*, 1340-1359.

169. Steiger, R.E. *J. Biol. Chem.* **1944**, *153*, 691-692.

170. Moller, B.L.; McFarlane, I.J.; Conn, E.E. *Acta Chem. Scand.* **1977**, *B31*, 343-344.

171. Chimiak, A.; Milewska, M.J. *Prog. in the Chem. of Org. Nat. Prod.* **1988**, *53*, 204-277.

172. Prepared by Dr Lister Lam by the method of Colonge, J.; Collomb, F. *Bull. Chem. Soc. Fr.* **1951**, 241-243.

173. Grewe, R. *Chem. Ber.* **1943**, *76*, 1072-1076.

174. Snoke, J.E. *J. Biol. Chem.* **1956**, *223*, 271-276.

175. Wong, C.-H.; Drueckhammer, D.G. *Bio/Technol.* **1985**, *3*, 649-651, and references cited therein.

176. Grisolia, S.; Quijada, C.L.; Fernandez, M. *Biochim. Biophy. Acta* **1964**, *81*, 61-70.

177. Bradford, M.M. *Anal. Biochem.* **1976**, *72*, 248-254.

178. Scopes, R.K. *Protein Purification: Principles and Practice;* 2nd Ed., Springer-Verlag: New York, 1987; pp 141-156.

179. Stellwagen, E. In *Guide to Protein Purification;* Deutscher, M.P., Ed.; Academic Press: San Diego, 1990; pp 343-357.

180. Biellmann, J.F.; Samama, J.P.; Branden, C.I.; Eklund, H. *Eur. J. Biochem.* **1979**, *102*, 107-110.

181. Scopes, R.K. *J. Chromatogr.* **1986**, *376*, 131-140.

182. Matsudaira, P. *J. Biol. Chem.* **1987**, *262*, 10035-10038.

183. Speicher, D.W. In *Techniques in Protein Chemistry;* Hugli, T., Ed.; Academic Press: New York, 1989; pp 24-35.

184. Segal, H.L.K.; Kachmar, J.F.; Boyer, P.D. *Enzymologia* **1952**, *15*, 187-198.

185. Cleland, W.W. *Meth. Enzymol.* **1979**, *63*, 103-138.

186. Velick, S.F.; Vavra, J. *J. Biol. Chem.* **1962**, *237*, 2109-2122.

187. Henson, C.P.; Cleland, W.W. *Biochemistry* **1964**, *3*, 338-345.

188. Boyde, T.R.C. *Biochem. J.* **1968**, *106*, 581-586.

189. Bulos, B.; Handlers, P. *J. Biol. Chem.* **1965**, *255*, 3487-3491.

190. Kiick, D.M.; Cook, P.F. *Biochemistry* **1983**, *22*, 375-382.

191. Henderson, P.J.F. In *Enzyme Assays: A Practical Approach*; Eisenthal, R.; Danson, M.J., Ed.; Oxford University Press: New York, 1992; Chapter 10.

192. Provided by Hoffmann-La Roche Inc., Nutley, N.J.

193. Perrin, D. D.; Armarego, W. L. F. In *Purification of Laboratory Chemicals*; Pergamon Press: Oxford, 1988.

194. Vedejs, E.; Engler, D.A.; Telschow, J.E. J. Org. Chem. 1978, 43, 188-196.

195. Brown, C.A. J. Org. Chem. 1974, 39, 3913-3918.

196. Krebs, K.B.; Heusser, D.; Wimmer, H. In *Thin-Layer Chromatography: A Laboratory Handbook*, 2nd Ed.; Stahl, E., Ed.; Springer-Verlag: New York, 1969; pp 854-909.

197. Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923-2925.

198. Fuji, K.; Nakano, S.; Fujita, E. *Synthesis* **1975**, 276-277.

199. Huynh, C.; Derguini-Boumechal, F.; Linstrumelle, G. *Tetrahedron Lett.* **1979**, *17*, 1503-1506.

200. Harmon, R.E.; Wellmann, G.; Gupta, S.K. *J. Org. Chem.* **1973**, *38*, 11-16.

201. Cason, J.; Reist, E.J. *J. Org. Chem.* **1958**, *23*, 1492-1495.

202. Jung, M.E.; Shishido, K.; Davis, L.H. *J. Org. Chem.* **1982**, *47*, 891-892.

203. Prepared by Dr Lister Lam according to the procedure in ref. 158. For an improved procedure see: Ireland, R.E.; Liu, L. *J. Org. Chem.* **1993**, *58*, 2899.

204. Prepared by Dr Lister Lam by the method of Abd El Samii, Z.K.M.; Al Ashmawy, M.I.; Mellor, J.M. *J. Chem. Soc. Perkin Trans. I* **1988**, 2517-2522.

205. Davis, B.D.; Mingioli, E.S. *J. Bacteriol.* **1950**, *60*, 17-28.